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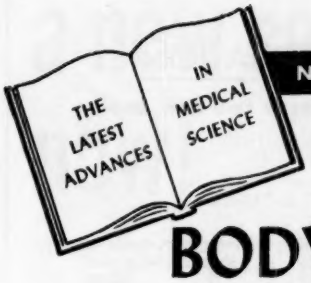

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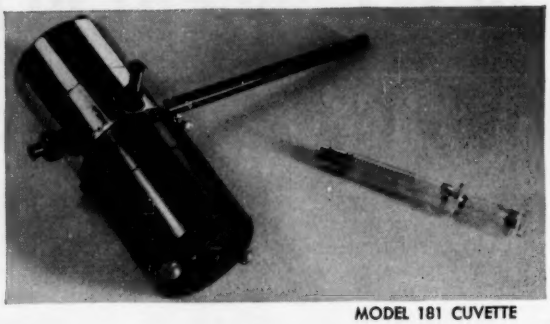

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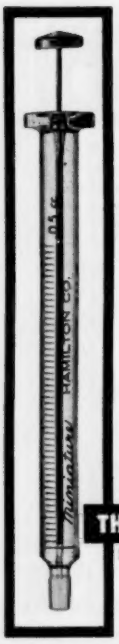

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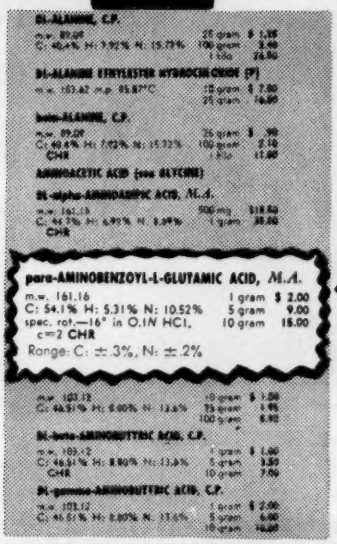

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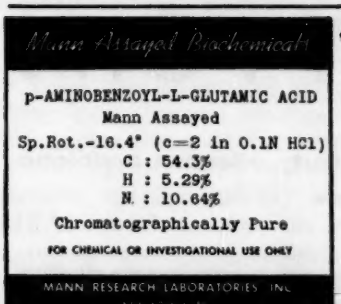

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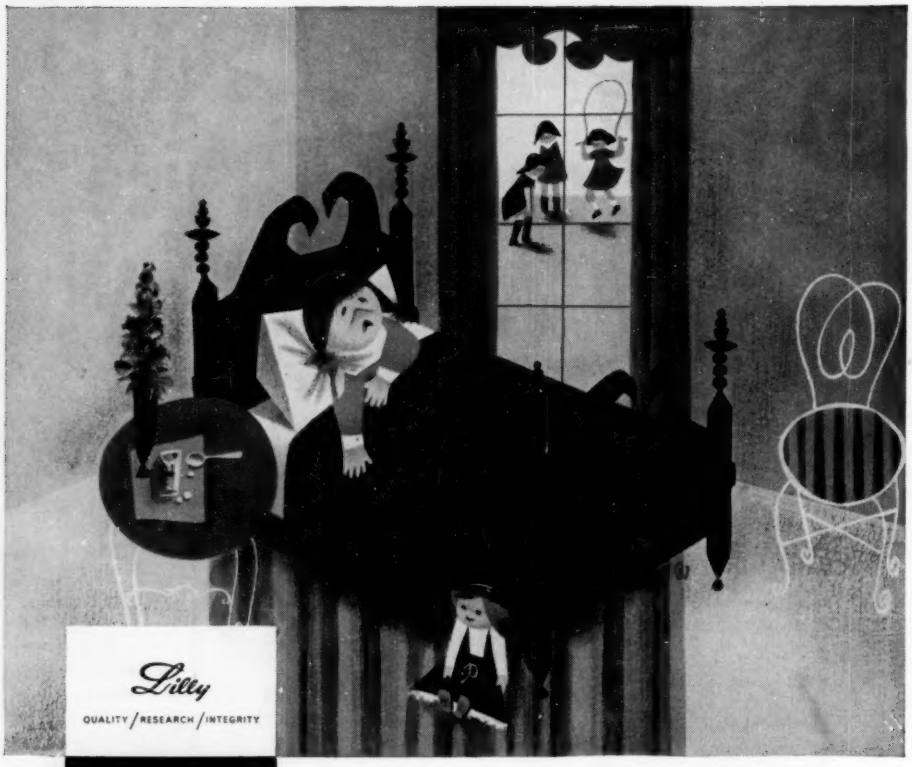

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GENERAL HEMODYNAMICS AND SPLANCHNIC CIRCULATION IN PATIENTS WITH COARCTATION OF THE AORTA [1]

By JAMES W. CULBERTSON, JOHN W. ECKSTEIN,[2] WALTER M. KIRKENDALL, AND GEORGE N. BEDELL [3]

(From the Hemodynamic Laboratory, Cardiovascular Research Laboratories [4] and the Department of Internal Medicine, College of Medicine, State University of Iowa, and the Medical Service, Veterans Administration Hospital, Iowa City, Iowa)

(Submitted for publication May 7, 1957; accepted July 11, 1957)

Controversy concerning the general hemodynamic pattern associated with coarctation of the aorta revolves primarily about the genesis of the systemic arterial hypertension, and the nature and distribution of the peripheral arterial resistance. It is generally agreed (2, 3) that both systolic and diastolic blood pressure levels are elevated above the site of constriction and that the systolic elevation is relatively greater than the diastolic, so that a wide pulse pressure is seen. It is further agreed (2, 3) that systolic and mean pressure levels in the femoral artery are lower than in the brachial artery and that femoral pulse pressure is small. Since there is a mean arterial pressure gradient between the proximal and distal portions of the aorta, two important propositions must be considered: a) if peripheral resistance is distributed uniformly throughout the body, there must be a relatively low blood flow rate through the aorta below the constriction; and b) if the blood flow rate is the same through the proximal and distal segment, the total resistance to flow from the distal segment must be relatively low or, conversely, the total resistance to flow from the proximal segment must be relatively high. Previous investigators have attempted to resolve these problems primarily by study of isolated segments of the circulation. Blumgart, Lawrence, and Ernstene (4) found normal arteriolar pressure in the arms and legs and suggested increased total arterial resistance in the upper part of the body. Prinzmetal and Wilson (5) and Pickering (6) supported this hypothesis by demonstrating that blood flow in the upper extremities was not increased. Steele (7) found elevation of femoral arterial diastolic pressure in three patients and felt that this was evidence for a generalized increase in arteriolar tone throughout the body in patients with coarctation of the aorta. He held that the notion of unequal distribution of the peripheral resistance was untenable because of these findings and a review of reports of observations on 217 patients described by other authors. Steele's argument, based on elevated femoral diastolic pressure, has been refuted by Hull (8), Bing and associates (2), and Gupta and Wiggers (9). These authors cite evidence to show that the tendency toward elevated femoral diastolic pressure is caused by damping of the pulse wave by the aortic constriction and the long collateral channels. It must be admitted that this explanation does not exclude the possible presence of generalized vasoconstriction in patients with coarctation. Such a notion might be supported by the work of Harris, Sealy, and DeMaria (3), who found elevated *mean* femoral arterial pressure in some patients. No generalization concerning widespread arteriolar constriction in the lower systemic arterial compartment seems justified in the absence of measurements of blood flow through the distal aorta. It seems unlikely, therefore, that problems regarding the general distribution of peripheral arterial resistance will be resolved completely until the rate of total blood flow through the lower arterial compartment is measured.

The hypertension in the upper arterial compartment has been attributed to a generalized systemic arteriolar constrictor influence such as that in "essential" or renal hypertension. This hypothesis has been advanced largely from the results

[1] Read by title at the 46th Annual Meeting of the American Society for Clinical Investigation, May 4, 1954 (1).

[2] Rockefeller Foundation Postdoctoral Fellow in the Medical Sciences (National Research Council).

[3] Postdoctoral Research Fellow of the National Heart Institute, Public Health Service.

[4] These laboratories are supported by the State University of Iowa, the Iowa State Department of Health, the Iowa Heart Association, and a cardiovascular training grant (HT-245) from the National Heart Institute of the Public Health Service.

of animal experiments (10–14) in which the production of renal ischemia by chronic aortic constriction has been followed by generalized hypertension. Despite its attractiveness, the analogy which has been drawn between the hypertension of experimental coarctation, experimental renal hypertension, "essential" arterial hypertension in man, and the hypertension associated with coarctation of the aorta in man likewise is not justified. Studies made on the renal circulation in clinical cases, although fraught with disagreement, have not demonstrated renal ischemia consistently. Some investigators (15, 16) have found the renal hemodynamic pattern to be similar to that seen in "essential" arterial hypertension. Others (3, 17), on the contrary, report normal renal plasma flow and glomerular filtration rates, as well as normal tubular mass (17). Some observers (4, 18) have held that the hypertension in the proximal aorta is caused by the mechanical obstruction to blood flow offered by the narrowed segment and the collateral vessels. An analogous form of hypertension can be produced in animals by acute aortic constriction (9, 10, 18–20). The postulation of a mechanical cause for the upper compartment hypertension has not been refuted directly.

Additional hemodynamic observations made on clinical cases of coarctation of the aorta have done little toward settling the controversy regarding the origin of the hypertension. Cardiac output has been found to be normal or increased (2, 21–23). Measurements have demonstrated total oxygen consumption to be normal (24) or increased (21, 25). Some investigators (26–28) have reported normal blood flow in the lower extremities, but others (2) have found it to be markedly diminished. Blood flow in the upper extremities has been reported variously as normal (6, 26, 28), increased (2), and decreased (5). Hafkenschiel, Crumpton, and Moyer (29) found increased cerebral blood flow and normal cerebral vascular resistance. These reports, sometimes conflicting or fragmentary, suggest that the hemodynamic pattern is not the same in all patients with coarctation of the aorta. It is reasonable to suppose that, if hemodynamic observations were made on vascular segments both above and below the coarctation at the same time or under the same experimental conditions in a given patient, more reliable interpretation of circulatory abnormalities might be possible. Therefore, we have studied brachial and femoral arterial pressure, minute volume cardiac output (= pulmonary blood flow rate ≒ total systemic blood flow rate), and the hepatic portal circulation in this manner in each of ten patients, with one exception in each of two of these functions. Additional observations were made on the renal circulation during separate experimental sessions in these ten and in eleven other patients. Summary renal circulatory data on the first ten patients only are included in this report for the purpose of general hemodynamic considerations. The detailed analysis of all our renal studies (30) will be presented in a separate communication.

MATERIAL AND METHODS

The usual criteria (31) for the diagnosis of coarctation of the aorta were present in every one of our ten patients: nine men and one woman (Ahl). None had clinical or laboratory evidence of valvular heart disease or additional congenital cardiovascular disorders. The diagnosis was confirmed at operation in all except Patient Wol, who did not receive surgical treatment; but his clinical and hemodynamic findings were unequivocal.

Experimental observations were made in the early morning with the patients in the fasting state and resting in the supine position. Phasic arterial pressure was measured with a capacitance manometer and registered on a multi-channel direct-writing oscillograph, and mean pressure values were obtained by electrical integration of the manometer output (Sanborn equipment). Great care was taken to establish the zero pressure baseline at the mid-atrial level.

Cardiac output was determined by means of the Fick principle with a catheter tip in the pulmonary artery, according to the method of Cournand and associates (32). Mixed venous blood was obtained from the pulmonary artery through the catheter, and systemic arterial blood was drawn simultaneously from the brachial artery through an indwelling needle. Manometric determinations of blood oxygen content were done as described by Van Slyke and Neill (33). Expired air was collected in a calibrated Tissot-type spirometer, and partial pressure of oxygen in these samples and in room air was measured with a Beckman Model C Oxygen Analyzer, checked periodically with the Haldane apparatus.

Hepatic blood flow was estimated by the method of Bradley, Ingelfinger, Bradley, and Curry (34) with certain modifications. Bromsulfalein was infused at approximately 2.5 mg. per minute per M.^{2} to allow calculation of the hepatic plasma clearance rate of bromsulfalein according to the method standardized by Culbertson and associates (35–38). Twenty minutes were allowed for equilibration after initial priming with 100 mg. of bromsulfalein. A peripheral arterial blood sample was drawn at the end of the equilibration period to establish the

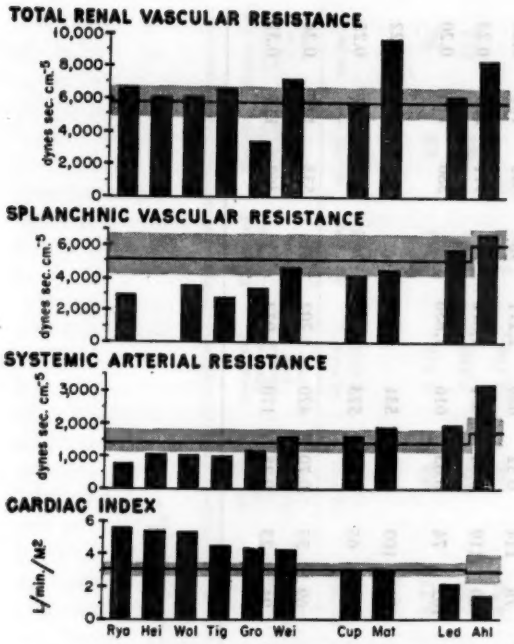

FIG. 1. CARDIOVASCULAR PARAMETERS IN PATIENTS WITH COARCTATION OF THE AORTA

The dark horizontal line in each frame represents an average normal value for that parameter. The width of the horizontal shaded area in the three lower frames is equal to two standard deviations. The offsets between Patients Led (male) and Ahl (female) represent sex differences. The normal values for cardiac index are taken from the observations of Cournand and co-workers (32) on 13 healthy men and six hospitalized women with normal circulatory systems. The normal values for systemic arterial resistance were calculated from the total blood flow data (cardiac index and surface area) from the same source, with an arbitrarily selected heart rate of 60 beats per minute (lower limit of normal)[6] and a mean blood pressure of 100 mm. Hg (average normal). The normal values for splanchnic vascular resistance were calculated in the same way using the data reported by Bradley and co-workers (34) from 18 men and five women, and the same arbitrarily selected pulse rate and blood pressure. The normal values for total renal vascular resistance were calculated according to the method of Gomez (42); the solid line and shaded area represent the average and range (rather than two standard deviations) from nine normal men and one normal woman in our laboratory, averaged without sex distinction.

[6] This tends to minimize the obvious difference between our coarctation patients and normotensive patients. Naturally, the difference between our patients and those with "essential" arterial hypertension is much greater (40). We realize that these are derived figures without the precision suggested by the unit dynes second cm.$^{-5}$ and could be expressed just as usefully as simple units of resistance representing the ratio of pressure to flow as recorded conventionally. The present notation has been used to facilitate comparison with other reported data.

initial peripheral bromsulfalein concentration. Thereafter, four sets of simultaneous peripheral arterial and hepatic venous blood samples were collected at ten-minute intervals. At twenty and at thirty minutes after equilibration simultaneous blood samples were drawn from these sites under oil to derive estimates of the splanchnic oxygen consumption in four of the patients. Bromsulfalein determinations were made on oxalated plasma. Blood volume was estimated from the nomograms of Gibson and Evans (39).

Renal plasma flow (para-aminohippuric acid) and glomerular filtration rate (inulin) were determined by the standard methods of Smith and associates (40–42).

Hemodynamic values were derived to permit comparison of the resistance to blood flow in various regional circuits. These are expressed in dynes cm. second^{-5}. The formulae used were as follows:

$$\text{Resistance to blood flow from the proximal aorta}[5] = \frac{\text{mean brachial arterial pressure, mm. Hg} \times 1332}{\text{cardiac output, ml. per sec.}};$$

$$\text{Splanchnic vascular resistance} = \frac{\text{mean femoral arterial pressure, mm. Hg} \times 1332}{\text{average estimated hepatic blood flow, ml. per sec.}}.$$

[5] "Systemic arterial resistance" in Figure 1. Total renal vascular resistance was calculated by the formulae of Gomez (42) and is equal to the sum of the afferent arteriolar, net efferent arteriolar, and venular resistance values. For estimations of splanchnic and renal vascular resistance mean femoral arterial pressure was used, except in the case of Patient Rya, in which mean brachial pressure was applied to the formulae. This obviously resulted in higher than the true values for this patient.

RESULTS

In the figure and tables the ten patients are arranged in order of descending magnitude of the cardiac index. In the first six patients this value was above normal (Figure 1 and Table I). Total oxygen consumption was increased in five patients; and the arteriovenous oxygen difference was diminished in four of these six cases, bearing in general an inverse relationship. The stroke volume cardiac output was elevated both absolutely and in relation to the heart rate in the first five. In the seventh and eighth patients the cardiac index, stroke volume, arteriovenous oxygen difference, and total oxygen consumption were normal. The final two patients were considered to be in

TABLE I

Summary of hemodynamic data

Patient Sex	Age	Surface area	Hematocrit	Brachial arterial pressure		Femoral arterial pressure		Pulmonary "capillary" mean* pressure	Total oxygen consumption rate	Pulmonary A-V oxygen difference	Cardiac index	Heart rate	Stroke volume	Average hepatic BSP extraction fraction	Average plasma BSP clearance rate	Average estimated hepatic blood flow rate	Average rate of splanchnic oxygen consumption	Average effective renal plasma flow rate	Average glomerular filtration rate	Average glomerular filtration fraction
				Phasic	Mean*	Phasic	Mean*													
	yrs.	*M.²*	*per cent*	*mm. Hg*	*mm. Hg*	*mm. Hg*	*mm. Hg*	*mm. Hg*	*ml./min.*	*ml./100 ml.*	*L./min./M.²*	*beats/min.*	*ml.*	*decimal*	*ml./min./1.73 M.²*	*ml./min./1.73 M.²*	*ml./min./1.73 M.²*	*ml./min./1.73 M.²*	*ml./min./1.73 M.²*	*decimal*
Rya M	24	1.94	44.5	155/90	105			11	347	3.2	5.57	52	208	0.67	950	2,568		631	128	0.20
Hei M	19	1.69	36.6	160/95	120	105/75	85		300	3.3	5.38	74	123		758			626	116	0.19
Wol M	44	1.98	44.1	205/100	135	115/80	95	7	339	3.2	5.35	73	145	0.50	538	1,997	86	624	142	0.23
Tig M	18	1.97	43.8	180/80	110	110/75	90	11	346	3.9	4.52	78	114	0.52	659	2,271	98	542	124	0.23
Gro M	18	1.88	44.0	165/90	115	120/75	85	11	288	3.5	4.36	69	119	0.63	651	1,870		971	226	0.23
Wei M	27	1.75	46.4	250/100	150	125/95	105	5	315	4.2	4.29	102	74	0.63	616	1,836		586	116	0.20
Cup M	44	1.93	45.9	165/90	120	105/80	85		265	4.5	3.06	54	109	0.64	531	1,460		553	121	0.22
Mat M	15	1.76	41.2	180/95	125	120/90	100	8	231	4.4	3.01	81	65	0.52	523	1,735	79	422	107	0.25
Led M	28	1.83	49.5	155/75	100	105/85	90	13	211	5.2	2.24	69	59	0.70	420	1,202	81	533	127	0.24
Ahl F	16	1.46	40.6	130/70	90	95/65	75	26	202	9.0	1.50	94	23	0.28	170	1,072		369	115	0.31

* Electrically integrated.

TABLE II

Observed hepatic circulatory and metabolic data *
(not corrected to standard surface area)

Patient and BSP load	Systemic arterial plasma BSP concentration	Hepatic venous plasma BSP concentration	Hepatic venous extraction fraction	Average intravenous infusion rate of BSP	Rate of change in systemic plasma BSP concentration	Estimated plasma volume	Removal rate of plasma BSP	Hepatic plasma BSP clearance rate	Estimated hepatic plasma flow rate	Estimated hepatic blood flow rate	Hepatic arteriovenous oxygen difference	Splanchnic oxygen consumption rate
mg./min./M.²	*mg./100 ml.*	*mg./100 ml.*	*decimal*	*mg./min.*	*mg./L./min.*	*liters*	*mg./min.*	*ml./min.*	*ml./min.*	*ml./min.*	*ml./100 ml.*	*ml./min.*
Rya 2.45	0.45	0.15	0.67	4.76	0.02	3.26	4.77	1,068	1,613	2,906		
Hei 2.34	0.53			3.96	0.01	3.19	3.94	743				
Wol 2.53	0.80	0.41	0.50	5.00	0.08	3.35	4.73	604	1,255	2,244	4.9	96
Tig 2.54	0.68	0.33	0.52	5.00	0.04	3.36	5.08	749	1,450	2,581	4.1	112
Gro 2.66	0.71	0.26	0.63	5.00	0.06	3.19	4.98	708	1,138	2,032		
Wei 2.40	0.71	0.27	0.63	4.20	0.07	2.81	4.40	622	994	1,855		
Cup 2.62	0.92	0.32	0.64	5.05	0.12	3.16	5.35	590	878	1,622		
Mat 2.50	0.80	0.39	0.52	4.40	0.06	3.11	4.26	534	1,040	1,770	4.2	81
Led 2.40	0.99	0.30	0.69	4.40	0.05	2.79	4.39	441	639	1,266	6.3	85
Ahl 2.44	2.32	1.68	0.28	3.56	0.15	2.26	3.31	144	540	909		

* Values for plasma concentration of bromsulfalein, extraction fraction, rate of change, removal rate, and the rates of plasma clearance, plasma flow and blood flow represent the means of four 10-minute observation periods. The infusion rate represents the mean of calculations from a calibrated infusion system drop count made every three minutes during each experiment. Estimated plasma volume is a single reading from the Gibson and Evans nomogram (39). Each value for arteriovenous oxygen difference and oxygen consumption rate is the average of two measurements. A tabular record of all of these original data has been deposited as Document No. 5285 with the ADI Auxiliary Publications Project, Photoduplication Service, Library of Congress, Washington 25, D. C. A copy may be secured by citing the Document No. and by remitting $1.25 for photoprints or for 35 mm. microfilm. Advance payment is required. Make checks or money orders payable to: Chief, Photoduplication Service, Library of Congress.

borderline (Patient Led) or subclinical (Patient Ahl) left ventricular failure on the basis of low cardiac output, normal oxygen consumption, high normal or large arteriovenous oxygen difference and elevated pulmonary "capillary" pressure. Study of these last two cases suggests that subclinical left ventricular failure may have been responsible for some of the discrepancies in values obtained for regional blood flow rates recorded in the literature without accompanying general hemodynamic data on patients with coarctation.

Hepatic blood flow (Tables I and II) was estimated in five of the first six patients and was found to be well above the normal average. The values tended to parallel the general systemic blood flow, as we have found in resting, fasting patients with other circulatory abnormalities (43, 44). The hepatic plasma clearance of bromsulfalein was generally normal, as was the hepatic extraction fraction of bromsulfalein. All of these hepatic circulatory and metabolic values were normal in the seventh and eighth patients but tended to be reduced in Patients Led and Ahl. Splanchnic oxygen consumption (Table I) in general paralleled hepatic blood flow but was not reduced in any of the four patients in whom it was measured.

In the brachial artery the systolic pressure was elevated in all patients not in heart failure, but the diastolic pressure was normal in six and only slightly increased in the remaining four (Table I). Pulse pressure generally was wide, in contrast to the finding in "essential" hypertension uncomplicated by structural arterial disease. Mean pressure was elevated considerably in one patient, moderately in one, and slightly in four; in four patients it was normal. Femoral artery systolic pressure was normal in each of nine recorded cases (not measured in Patient Rya). Femoral diastolic and mean pressure levels were normal, except for Patient Wei, in whom the diastolic pressure was slightly increased. The femoral arterial pulse pressure was characteristically narrow.

Renal plasma flow and glomerular filtration rates (Table I) fell generally within the normal range in the first seven patients. We cannot explain the unusually high values in Patient Gro (in this patient, measurements made at about six months after operation revealed lower but still abnormally high values in spite of a modest fall in cardiac output). A slight elevation in filtration fraction was noted in four of these first seven patients. The renal plasma flow was definitely low in Patient Mat, and there was a distinct increase in his filtration fraction. The renal pattern in the two patients in borderline or subclinical heart failure (Patients Led and Ahl) was characterized by the expected reduced plasma flow, normal glomerular filtration, and increased filtration fraction.

DISCUSSION

If the hypertension in clinical coarctation of the aorta is similar to that observed in "essential" arterial hypertension in man, then all organs and tissues in the body which receive blood from the systemic arterial compartment should share in increased peripheral arteriolar resistance. To refute the argument that the hypertension associated with coarctation of the aorta is similar to "essential" or renal hypertension, the investigator must demonstrate the absence of a generalized increase in the peripheral resistance or show some gross inequality in its distribution.

In seven of our eight patients without evidence of myocardial decompensation the relationship between cardiac output and mean brachial arterial pressure was such that overall resistance to blood flow from the proximal aortic compartment was normal or below normal (Figure 1). A similar pressure-flow relationship was evident in the splanchnic circuit, where not one of nine had an increased arteriolar resistance, in sharp contrast to the finding in "essential" hypertension (45, 46). The situation in the kidney was somewhat different. Renal plasma flow, although generally within normal limits, tended to be relatively low. Because of this, estimates of total renal vascular resistance were relatively high when compared to the hepatic circuit, except for Patient Gro with the renal hyperemia. Of the patients not in heart failure only Patient Mat exhibited a significantly low renal plasma flow. In this case alone the renal vascular resistance approached levels seen in established "essential" hypertension, although splanchnic resistance remained relatively low, in contrast to the characteristic finding in "essential" hypertension.

Our data fail to show increased resistance to blood flow out of the proximal aorta, or through the kidney, or in the splanchnic vascular bed in

patients not in myocardial decompensation. They do indicate that the peripheral resistance has gross inequalities in its distribution in patients with coarctation of the aorta not in congestive heart failure, and that resistance may be decreased or normal in vascular circuits both above and below the constriction in the same patient. There appeared to be a definite tendency for splanchnic vascular resistance to parallel resistance to blood flow from the proximal aortic segment. No such relationship appeared to exist between the renal and the splanchnic circuits, or between the renal circuit and the upper aortic compartment. This suggests that local regulatory mechanisms, rather than a generalized vasoconstrictor influence, are responsible for the distribution of the peripheral resistance, at least with respect to the renal circuit. In no patient, including Patient Ahl, who was in early congestive heart failure, did we find evidence of the generalized increase in arteriolar resistance which characterizes patients with "essential" systemic arterial hypertension.

The demonstration of a wide pulse pressure in the brachial artery, along with an increased stroke volume in the majority of our patients, is similar to the observations of Gupta and Wiggers (9) in dogs. Our findings support their suggestion that the hypertension above the stricture is related to the degree of narrowing, to a foreshortening of the aortic compression chamber, and to an increased stroke volume of the left ventricle. We believe that these observations on pulse pressure and stroke volume, plus those on the unequal distribution of the peripheral resistance, and the evidence against renal ischemia in the majority of our patients form strong evidence against a renal mechanism in the genesis of the upper compartment systemic arterial hypertension seen in coarctation of the aorta.

CONCLUSIONS

1. The hemodynamic pattern associated with coarctation of the aorta in ten patients is in sharp contrast to that observed in "essential" systemic arterial hypertension or renal hypertension in man.

2. The hypertension observed in the upper arterial compartment should be explained on the basis of mechanical factors and local regulatory mechanisms rather than a postulated generalized vasoconstrictor influence.

3. Evidence for renal ischemia was found in only one of eight patients who were free of congestive heart failure; no hepatic ischemia was seen in the nine patients in whom hepatic blood flow was measured, despite the presence of subnormal mean pressure levels in the lower systemic arterial compartment. The tendency was toward splanchnic hyperemia.

4. The demonstration of incipient or subclinical heart failure in two of our ten patients suggests a possible explanation for some of the discrepancies among previously published reports on isolated hemodynamic features of coarctation of the aorta.

ACKNOWLEDGMENT

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THE POTENTIATION OF EXOGENOUS INSULIN BY TOLBUTAMIDE IN DEPANCREATIZED DOGS[1]

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The mode of action of the hypoglycemic sulfonylureas has remained obscure despite the great interest and extensive clinical trial of these agents in the treatment of diabetes mellitus. A thorough understanding of the mechanism of action is essential in view of their potentially extensive use in diabetes.

Several modes of action have been suggested: a) inhibition of the pancreatic alpha cells resulting in a decreased production of glucagon (1); b) increased insulin production due to stimulation of the pancreatic beta cells (2); c) inhibition of insulinase resulting in decreased inactivation of insulin (3); and d) inhibition of one or more enzymes of the liver resulting in decreased hepatic glucose output (4–6).

It is generally agreed by most investigators that the sulfonylureas do not affect the alpha cells and so do not inhibit glucagon secretion (6, 7). Additional evidence negating this theory is the absence of hypoglycemic action in completely alloxanized animals (8, 9).

Vaughan (5) has reported that the hypoglycemia may be due to inhibition of one or more of the liver enzymes resulting in diminished glucose output. If this were so, one would expect hypoglycemic action in depancreatized and alloxanized animals. This does not occur.

The beta cell stimulation (2) and insulin potentiation theories (3, 10, 11) are the two most generally considered mechanisms of action. We have sought to clarify this problem by determining whether or not tolbutamide (Orinase®)[2] could enhance the action of insulin in the absence of all pancreatic tissue. If this occurred, it would mean the mode of action (or at least one of the mechanisms) was to potentiate insulin activity since the animals had no beta cells and so lacked a source of endogenous insulin. This work is a report of the effect of tolbutamide on the insulin tolerance tests of depancreatized dogs.

METHODS

Mongrel male and female dogs were pancreatectomized with utmost care to assure complete removal of all pancreatic tissue. They were maintained on milk, canned dog food, and regular dog meal, and were allowed a one-week recovery period during which 8 units of NPH insulin were given daily. From then on the animals received 5 units of regular insulin once per day. This was given 18 hours prior to the experiment of the following day.

After the seven-day recovery period, insulin tolerance

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[2] Trade name for tolbutamide, The Upjohn Co.

TABLE I

The effect of tolbutamide on the insulin tolerance tests of depancreatized dogs using 0.25 unit per Kg. of insulin

Dog no.	Total insulin units	Tolbutamide	Control period	Blood glucose (Hours) 0	½	1	2	3	4
		mg./Kg.	*mg. %*			*per cent change*			
1	3.7	0	266	0	−21.0	−41.0	−61.0	−52.5	−40.0
	3.7	100	248	0	−30.2	−58.0	−69.0	−60.5	−52.2
2	2.8	0	325	0	−23.0	−38.5	−43.0	−38.5	−37.0*
	2.8	100	297	0	−35.0	−49.4	−58.2	−57.0	−48.3*

* Three and one-half hours.

TABLE II

The effect of tolbutamide on the insulin tolerance tests of depancreatized dogs using 0.20 unit per Kg. of insulin

Dog no.	Total insulin units	Tolbutamide	Control period	Blood glucose (Hours) 0	½	1	2	3*	4†
		mg./Kg.	*mg. %*	*per cent change*					
2	2.2	0	358	0	−30.2	−43.8	−52.0	−38.8	−18.7
	2.2	0	334	0	−14.4	−31.8	−44.5	−28.8	−11.7
	2.2	100	326	0	−30.0	−47.4	−57.5	−65.7	−67.2
4	2.1	0	368	0	−19.1				− 9.0
	2.1	0	354	0	−14.4	−30.3	−31.5	−26.6	− 6.5
	2.1	100	386	0	−25.4	−44.6	−65.0	−54.5	−44.6
7	1.8	0	365	0	−18.9	−23.9	−32.1	−23.9	−20.0
	1.8	0	334	0	−11.4	−20.4	−27.7	−20.4	0.0
	1.8	100	400	0	−15.5	−32.0	−46.3	−38.8	−35.8

* Mean ± SE {control, 27.7 ± 3.1. test, 53.0 ± 7.8 ($0.02 > p > 0.01$).

† Mean ± SE {control, 10.9 ± 3.1. test, 48.1 ± 9.4 ($0.01 > p > 0.001$).

tests were performed with varying doses of regular insulin (0.01, 0.025, 0.20, 0.25 units per Kg.). Following an overnight fast, a blood specimen was obtained from a limb vein, and the calculated quantity of insulin was administered intravenously in a single injection requiring five to ten seconds. Specimens of venous blood were obtained at zero, one-half, one, two, three and sometimes four hours. Following completion of the test the animals were given their maintenance dose of 5 units of regular insulin and were fed.

On the following day the test was repeated on the same animal using the same dose of insulin. However, one hour prior to the administration of insulin, the animal was given the sodium salt of tolbutamide (100 mg. per Kg.) intravenously dissolved in 4 ml. normal saline. Blood samples were then obtained at zero, one-half, one, two, three and sometimes four hours. In several cases control insulin tolerance tests were repeated on two successive days before performing the tolbutamide-insulin test. This was done to make certain of the constancy of the control curves, thus ruling out any artifact which might arise from performing the control test first. After once having received tolbutamide, the dog was not used again for at least five days to make certain all the sulfonylurea had been excreted.

In order to make certain of the completeness of the pancreatectomy in each dog, tolbutamide was injected intravenously (100 mg. per Kg.) 18 hours after the last dose of regular insulin. Blood glucose was determined at zero, one-half, one, two and three hours in two animals and at zero and one hour in all the others. All animals were fasted overnight. As an additional check on the completeness of the pancreatectomy, one-third of the animals were sacrificed and examined post mortem. No pancreatic tissue was found in any animal examined.

The animals were not anesthetized and were secured with a leash to prevent them from moving about during the experiment.

Blood glucose was determined by the Nelson modification of the Somogyi method (12).

TABLE III

The effect of tolbutamide on the insulin tolerance tests of depancreatized dogs using 0.025 unit per Kg. of insulin

Dog no.	Total insulin units	Tolbutamide	Control period	Blood glucose (Hours) 0	½	1*	2†	3‡
		mg./Kg.	*mg. %*	*per cent change*				
2	0.225	0	296	0	− 2.0	− 4.7	− 9.5	− 4.7
	0.225	100	277	0	−19.2	−31.4	−29.6	−26.0
7	0.170	0	350	0	0.0	+ 2.9	+ 7.1	+14.2
	0.170	100	372	0		−10.5	− 3.8	− 2.7
8	0.360	0	281	0	− 3.2	− 8.2	− 5.4	+ 1.8
	0.360	100	276	0	− 6.9	−13.5	−18.8	−13.8
10	0.250	0	280	0	− 1.9	− 3.5	0.0	+ 5.3
	0.250	100	276	0		−15.6	−13.8	−13.8
13	0.220	0	296	0	− 1.9	− 5.1	− 3.5	− 2.0
	0.220	100	357	0	− 6.5	−14.6	−17.1	−14.6

* Mean ± SE {control, −3.7 ± 1.8. test, −17.1 ± 3.7 ($0.02 > p > 0.01$).

† Mean ± SE {control, −2.2 ± 2.8. test, −16.0 ± 4.2 ($0.02 > p > 0.01$).

‡ Mean ± SE {control, +2.91 ± 3.1. test, −14.2 ± 3.7 ($0.01 > p > 0.001$).

TABLE IV

The effect of tolbutamide on the insulin tolerance tests of depancreatized dogs using 0.01 unit per Kg. of insulin

Dog no.	Total insulin units	Tolbutamide	Control period	Blood glucose (Hours) 0	½	1	2	3
		mg./Kg.	*mg. %*	*per cent change*				
10	0.10	0	214	0	+5.8	+3.7	+5.8	+ 9.2
	0.10	100	277	0	−6.9	−3.6	−3.6	− 5.4
11	0.09	0	246	0	−2.0	+9.0	0	+11.4
	0.09	100	277	0	−6.9	−5.4	0	0

RESULTS

The data are expressed as per cent change from the control blood glucose value rather then in absolute figures. This permitted comparison from animal to animal regardless of the difference in the control values.

The effect of tolbutamide on the insulin tolerance tests of depancreatized dogs using 0.25 unit per Kg. of insulin is shown in Table I. During the four hours of observation no significant effect was seen.

When the insulin dosage was reduced to 0.20 unit per Kg., (Table II), a potentiating effect by tolbutamide became evident during the third and fourth hours. When submitted to statistical analysis the difference was found to be significant.

When 0.025 unit per Kg. of insulin was used (Table III), augmented insulin action was apparent in one-half hour, well established by the end of the first hour, and continued during the three hours of observation. Thus, the smaller the quantity of insulin used, the earlier the potentiating effect of tolbutamide become evident.

Two dogs were given 0.01 unit per Kg. of insulin (Table IV). The dosage was so small that no hypoglycemic effect occurred in the control tests. However, when tolbutamide was administered, the same amount of insulin caused a maximal fall of blood glucose in one-half hour and a mild depression which persisted for three hours in Dog 10 and for two hours in Dog 11.

The effect of intravenous tolbutamide on the blood glucose of depancreatized dogs in the absence of any insulin is shown in Table V. Tolbutamide alone did not depress the blood glucose of any animal used in this work.

TABLE V

The effect of intravenous tolbutamide (100 mg. per Kg.) on blood glucose of totally depancreatized dogs in the absence of exogenous insulin

Dog no.	Blood glucose (Hours) 0	1	2	3
	mg. per 100 ml.			
2	277	277		
2	326	326		
4	350	386		
7	380	400		
8	272	276		
8	278	282	314	292
8	264	284		
10	258	277		
10	260	260		
11	258	277		
11	280	310		
13	282	314	310	310
13	358	367		

DISCUSSION

The data show that tolbutamide potentiated the action of exogenous insulin in the absence of all pancreatic tissue. Since the depancreatized animal no longer possessed beta cells and so lacked a source of endogenous insulin, one must conclude that the enhanced hypoglycemic effect of the insulin was due to potentiation by tolbutamide.

The inability of intravenous tolbutamide to lower the blood glucose in each animal when insulin was not administered showed that there was not enough endogenous insulin present to lower the blood glucose.

The apparent absence of insulin potentiation by tolbutamide during the first two hours when relatively large amounts of insulin were used may have been due to the fact that there was such an excess of insulin that its hypoglycemic action concealed or overshadowed the augmenting effect of the sulfonylurea. However, as more time elapsed and the concentration of the insulin diminished, the

augmenting effect of tolbutamide became evident. Consequently, the smaller the insulin dosage, the earlier the sulfonylurea effect occurred; and, the larger the insulin dosage, the longer the time required to demonstrate the potentiation.

Fritz, Morton, Weinstein, and Levine (13) were unable to demonstrate potentiation of exogenous insulin activity by carbutamide in depancreatized dogs maintained on constant intravenous infusions of insulin. These were almost the same experiments that we performed but with one significant difference. Insulin was administered in a continuous infusion during a three- to four-hour period, whereas we gave the insulin in a single injection requiring only a few seconds. By providing a constant and continuous source of insulin an excess was always maintained so that the concentration never fell as in our experiments. Thus the potentiating effect of the carbutamide was overshadowed and never observed during the course of the experiment by these investigators. Another way of expressing the above is that their animals were always maintained under the conditions of the first two hours of our higher insulin dosage experiments. This factor may account for the conflicting results of different investigators regarding potentiation of exogenous insulin by the sulfonylureas.

This work showed that one of the mechanisms of tolbutamide action was to enhance both the intensity and duration of insulin action. Prolonging the duration of action appears to be the more important of the two. The maximal augmentation of intensity did not appear to be very large. However, by prolonging the duration of action, this agent probably achieves most of its clinical effect.

Although insulinase inhibition is one way of accounting for insulin potentiation by the sulfonylureas, it is not necessarily the only explanation. Augmented insulin action could occur by some other unexplained mechanism. For example, it is conceivable that endogenous and extracted insulin could differ in some unknown manner and because of this difference the sulfonylureas potentiate the former, and have a much smaller effect on extracted insulin. This explanation would best fit all the known facts of sulfonylurea hypoglycemic action. Very little is known about insulin differences; therefore, the above is merely speculation. It does, however, serve to show that insulin potentiation might be explained without invoking the inhibition of insulinase.

The facts which have been marshalled in favor of the beta cell stimulation theory also support the insulin potentiation theory. Failure to lower blood glucose in the depancreatized animal can be explained by the absence of insulin as well as by the absence of beta cells. Since insulin is not available in the depancreatized or completely alloxanized animal, no potentiation of insulin activity can be expected. The supposed inability of the sulfonylureas to reduce the insulin requirement of totally depancreatized people (14) and juvenile diabetics (15) is given as evidence against the insulin potentiation concept. However, various investigators have reported a lowering of the insulin requirement of juvenile diabetics (16) and depancreatized dogs (17).

These substances probably have more than one mechanism of action. Most experimental facts to date favor the combination of insulin potentiation (endogenous and/or exogenous) and beta cell stimulation. Thus, tolbutamide probably exerts a true insulin effect, although indirectly, despite conflicting reports regarding peripheral insulin-like action.

SUMMARY

1. Tolbutamide (Orinase®) was found to potentiate the action of exogenous insulin in the absence of all pancreatic tissue.

2. One mechanism of tolbutamide action was to augment both the intensity and duration of insulin activity. Prolonging the duration appears to be the more important of the two.

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A DUAL MECHANISM OF VITAMIN B_{12} PLASMA ABSORPTION [1]

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Recent reports have described two types of plasma absorption curves following the oral administration of vitamin B_{12}. With microbiologic assay methods, after massive doses of vitamin B_{12}, early significant plasma levels have been found indiscriminately in both pernicious anemia patients and control subjects (1, 2). On the other hand, two independent laboratories, using radio-labeled cyanocobalamin, have successfully determined plasma absorption curves after the oral administration of only 0.46 to 1.0 microgram doses (3, 4). With these small amounts of vitamin B_{12} a distinctly different type of absorption curve was found in normal subjects and in pernicious anemia patients when intrinsic factor was added. This curve was characterized by little or no plasma radioactivity during the first four hours of the test and a peak concentration in the 8- to 12-hour interval. Furthermore, there was clear differentiation between control subjects and patients with pernicious anemia, because at these dosage levels the latter without added intrinsic factor showed insignificant plasma radioactivity.

In this investigation plasma absorption curves were obtained in pernicious anemia patients with and without intrinsic factor, as well as in control subjects after the oral administration of test doses of radio-labeled cyanocobalamin which ranged from 0.56 to 500 micrograms. This included a dosage range not previously examined.

MATERIAL AND METHODS

Cobalt^{58}-labeled vitamin B_{12} [2] used in test doses had an initial specific activity of 1,137 microcuries per mg., while that of the Co^{60}-labeled vitamin B_{12} [2] used was 893 microcuries per mg. Crystalline non-radioactive vitamin B_{12} was added as necessary to make up the desired test dosages. In each case the test dose was administered in approximately 100 ml. of water after an overnight fast; breakfast was withheld for two hours. When used, intrinsic factor concentrate (IFC) [3] was added in 100- or 200-mg. amounts to the solution of cyanocobalamin. This material had been found active in pernicious anemia patients when tested by the methods of Heinle, Welch, Scharf, Meacham, and Prusoff (5) and that of Schilling (6). Blood was withdrawn at different time intervals after the test. Radioactivity measurements [4] were made as detailed elsewhere (3). Briefly, a 20-ml. well-type scintillation counter was used with an automatic vial sample-changer and a recording timer. The overall efficiency of this counter was 18.4 per cent for Co^{58} and 24.0 per cent for Co^{60}. A pre-set count of 32,000 was used for each determination in this study. At this count, three times the standard deviation of the background was five counts per minute (cpm). Generally, in each study a pre-test plasma sample was used for the background count. Altogether, eight tests were carried out in eight control subjects and 12 tests in eight patients with pernicious anemia in remission. The diagnosis in each of the latter had been substantiated by absorption tests with and without the addition of IFC.

External monitoring over the liver area was carried out one to two weeks after the test dosages according to the method of Glass, Boyd, Gellin, and Stephanson (7) with the use of a NaI (thallium-activated) probe scintillation counter. The crystal, 1½ inches in diameter and 1 inch in thickness, was mounted on an RCA 5819 photomultiplier tube and shielded by ¾ inch of lead. Ten-minute counts were registered on a Tracerlab Superscaler, model SC 18A.

The amounts of vitamin B_{12} absorbed into the plasma were calculated from the radioactivity measurements with the following formula: micromicrograms of vitamin B_{12} per ml. of plasma equal pr/20 mk; where p is the oral dose of vitamin B_{12} in micromicrograms; r is the experimental net cpm in 20 ml. of plasma; m is the amount of radioactivity in the test dose in microcuries; k is the overall efficiency of the counter times disintegrations per minute per microcurie (2.22×10^6). This calculation was based upon the assumption that the radioactivity represented

[1] This investigation was supported by a grant from Eli Lilly and Company to the University of Minnesota.

[2] Kindly supplied by Dr. N. S. Ritter, Merck and Co., Rahway, N. J.

[3] Kindly supplied by Dr. R. W. Heinle, The Upjohn Company, Kalamazoo, Michigan.

[4] Facilities of the Radioisotope Unit of the Minneapolis Veterans Administration Hospital were used for radioactivity measurements.

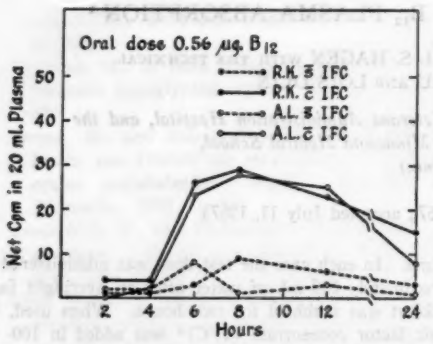

FIG. 1. RADIOACTIVITY OF PLASMA IN TWO PATIENTS WITH PERNICIOUS ANEMIA IN REMISSION AFTER THE ORAL ADMINISTRATION OF 0.56 MICROGRAM VITAMIN B_{12} CONTAINING 0.5 MICROCURIE Co^{60} WITH AND WITHOUT INTRINSIC FACTOR CONCENTRATE (IFC)

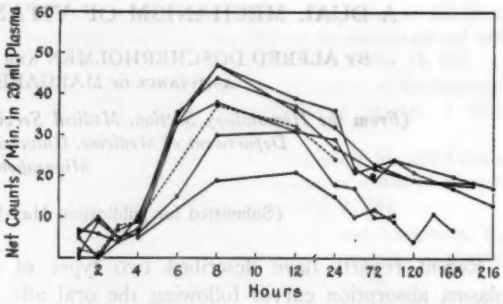

FIG. 2. RADIOACTIVITY OF PLASMA IN SIX CONTROL SUBJECTS (SOLID LINES, TEST DOSE 0.46 MICROGRAM VITAMIN B_{12}, 0.5 MICROCURIE Co^{60}) AND IN ONE PATIENT WITH LAENNEC'S CIRRHOSIS AFTER A PORTACAVAL SHUNT OPERATION (BROKEN LINE, TEST DOSE 0.56 MICROGRAM VITAMIN B_{12}, 0.5 MICROCURIE Co^{60})

cyanocobalamin and not a split-off cobalt salt. This was a reasonable presumption because the radioactivity is stored in the same way after oral and parenteral administration of Co^{60}-labeled vitamin B_{12}, but in a completely different manner from that observed after parenterally injected $Co^{60}Cl_2$ (8).

In a few instances the large oral doses of vitamin B_{12} contained radioactivity in amounts above those calculated to be safe (Co^{58}, 8 microcuries; Co^{60}, 3 microcuries) if entirely absorbed. This was justifiable because only a small fraction of these test doses was expected to be absorbed (9). Moreover, the highest liver radioactivity found in any of the patients in this study was within the general range reported by others after Co^{60} (7, 10).

RESULTS

Two male patients with pernicious anemia, aged 61 and 64, were given oral test doses of 0.56 microgram (0.5 microcurie Co^{60}), first without and later with the addition of 100 mg. IFC. Four weeks elapsed between the repeated tests. Figure 1 shows that there was but little radioactivity in the plasma unless intrinsic factor was given with the test dose. When IFC was added, absorption curves were obtained similar to those found in control subjects given test doses of like amount (3) (Figure 2); negligible radioactivity appeared in the plasma during the first four hours of the test, and peak concentrations in the plasma were not attained until eight hours following dosage.

Differing absorption curves were observed in five of six pernicious anemia patients given from 50- to 300-microgram doses of radio-labeled vitamin B_{12} without IFC. The results are summarized in Table I and are shown graphically in Figure 3. One patient with the 50-microgram test dose had little plasma radioactivity, but each of the other five had relatively high values by three hours with peak concentrations usually in four to six hours.

Two of these patients were retested with the same doses of vitamin, but with the addition of 200 mg. IFC. The early rise in plasma radioactivity was abolished in each case (Figure 4). Instead there was a gradual rise in radioactivity to peak values at 12 hours. While there was inhibition of that phase of absorption not mediated by intrinsic factor, radioactivity measurements over the livers

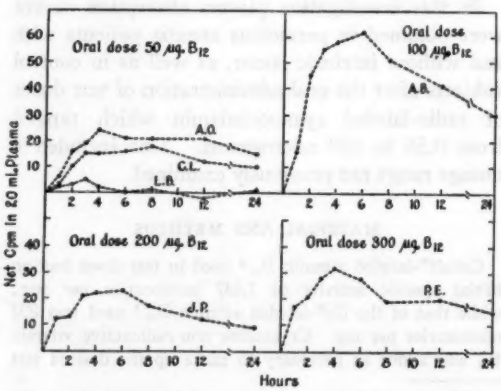

FIG. 3. RADIOACTIVITY OF PLASMA IN SIX PATIENTS WITH PERNICIOUS ANEMIA AFTER THE ORAL ADMINISTRATION OF 50 TO 300 MICROGRAMS OF RADIOACTIVE VITAMIN B_{12}

TABLE I

Radioactivity (cpm) in 20 milliliters of plasma at different time intervals after the oral administration of various test doses of Co^{58} vitamin B_{12}

Name*	Age	Diagnosis	Vit. B_{12} μg.	Radioactivity μc.	Cpm at different hours after oral test dose											Max. calculated amount labeled vitamin B_{12} μμg./ml. plasma	Radioactivity over the liver net cpm
					1	2	3	4	5	6	8	10	12	24	36		
H. M.	23	No disease	10	5.0	8	8	8	13		38	66		56		31	16	
G. B.	21	No disease	50	8.0	7	9	11	11	15	25	34	32	29	23		26	1,125
R. R.	40	Psychoneurosis	50	8.0	3	7	6	9	15	23	24			16		18	
E. J.	25	Appendicitis	100	8.0	1	2	4	9	12	14	16		14	11		24	
E. E.	59	Ess. hypertension	100	8.0	13	9	17	20		20	34		23	21		51	
A. H.	58	Emphysema	200	16.0	11	15	19	26		27	36		33	26		54	1,123
M. F.	44	Duodenal ulcer	300	16.0	14	21	20	17	20	22	25		27	21		61	
G. G.	40	Br. asthma, diabetes	500	16.0	7	15	19	18	19	22	21		21	19		83	
A. O.†	60	Pernicious anemia	50	8.0	6	16	19	24		21	21		21	15		18	670
L. B.†	63	Pernicious anemia	50	8.0	2	3	5	2		0	1		−2	1		4	118
C. L.†	64	Pernicious anemia	50	8.0	3	10	15	15		16	18		12	10		14	254
C. L.†‡	64	Pernicious anemia	50	8.0	2	3	4	6		8	14		21	14		16	721
A. B.	68	Pernicious anemia	100	9.8	16	45	55	58		62	51		41	30		76	760
A. B.‡	68	Pernicious anemia	100	8.9	5	4	6	10		15	20		22	11		30	946
J. P.	45	Pernicious anemia	200	16.0	12	13	21	22		23	17		12	9		35	170
P. E.	66	Pernicious anemia	300	16.0	17	21	28	32		28	19		20	15		72	370

* All were male patients.
† Approximately 4.0 microcuries CO^{60} B_{12} included.
‡ Two hundred mg. intrinsic factor concentrate added.

after IFC showed higher counts in each instance (Table I).

Figure 5 depicts the absorption curves in eight control subjects given 10- to 500-microgram test doses of radio-labeled cyanocobalamin. Three kinds of curves were obtained: the familiar delayed type; a new, somewhat biphasic, plateau-shaped configuration; and curves intermediate between these two types. None of the curves were the same as that found with comparable dosages of vitamin without addition of IFC in patients with pernicious anemia. The early, proportionately large amount of radioactivity in the plasma found in pernicious anemia was not obvious until the 200- to 300-microgram dosage. Then, in contrast to the con-

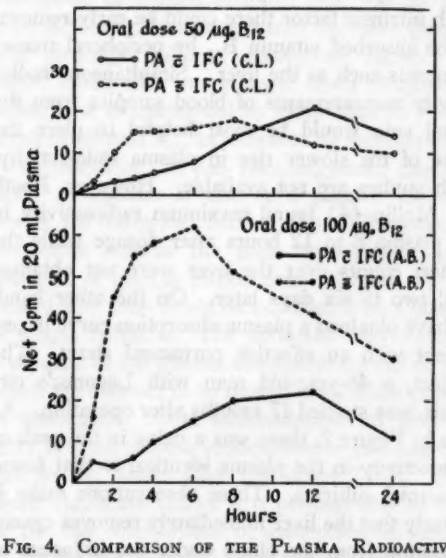

FIG. 4. COMPARISON OF THE PLASMA RADIOACTIVITY IN TWO PATIENTS WITH PERNICIOUS ANEMIA AFTER THE ORAL ADMINISTRATION OF 50 AND 100 MICROGRAMS OF RADIOACTIVE VITAMIN B_{12} WITH AND WITHOUT INTRINSIC FACTOR CONCENTRATE (IFC)

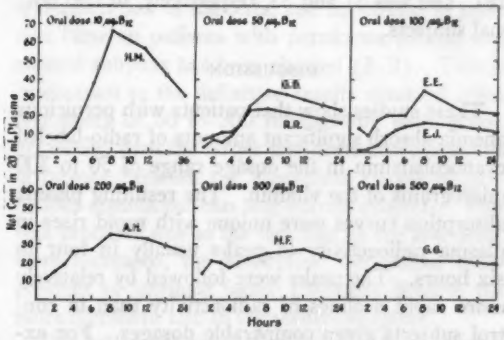

FIG. 5. RADIOACTIVITY OF PLASMA IN EIGHT CONTROL SUBJECTS AFTER THE ORAL ADMINISTRATION OF 10 TO 500 MICROGRAMS OF VITAMIN B_{12} CONTAINING 5.0 TO 16.0 MICROCURIES OF Co^{58}

tinued fall-away after the early peak in the pernicious anemia patients, a second phase was observed in the control subjects, in which the plasma radioactivity increased and persisted for many hours. At dosage levels of 50 to 100 micrograms intermediate types of curves were found.

Table I includes the maximum amounts of vitamin B_{12} absorbed into the plasma. Sixteen micromicrograms per ml. of plasma were found when the 10-microgram test dose was given to a control subject. In general, with increasing size of test doses larger amounts of cyanocobalamin were found, 83 micromicrograms per ml. being observed in the subject who received the 500-microgram test dose. This same tendency was seen in the six pernicious anemia patients to whom IFC was not given. However, the individual results showed wide variation.

The results of measurements of radioactivity over the liver one to two weeks after dosage are recorded in Table I. Consistently higher values were obtained in normal subjects and in patients with pernicious anemia when IFC accompanied the test dose, than in patients with pernicious anemia without IFC. This was true even when higher peak plasma radioactivity had been found in the latter. The effect of intrinsic factor can be illustrated by comparison of ratios of liver radioactivity to the peak radioactivity of 20 ml. of plasma. The average ratio was 16 in six patients with pernicious anemia without the addition of IFC to the test dose (range, 7 to 28). On the other hand, the ratio was raised to 34 and 43 in two pernicious anemia patients retested with added IFC, and was 31 and 33, respectively, in two normal subjects.

DISCUSSION

These studies show that patients with pernicious anemia absorb significant amounts of radio-labeled cyanocobalamin in the dosage range of 50 to 300 micrograms of the vitamin. The resulting plasma absorption curves were unique with rapid rises in plasma radioactivity to peaks usually in four to six hours. The peaks were followed by relatively more rapid declines of radioactivity than in control subjects given comparable dosages. For example, in the pernicious anemia patients the average plasma concentration after 24 hours was 45.4 per cent of the peak plasma level, compared to 71.6 per cent in control subjects. The difference is statistically significant, $t = 3.9$, $p < 0.01$.

Of particular interest was the finding that IFC abolished this early type of absorption curve and gave rise instead to gradual increases of radioactivity in the plasma with delayed peaks (Figure 4) such as found with smaller test doses with intrinsic factor present. Although excessive amounts of intrinsic factor can inhibit the absorption of small test doses (*e.g.*, 0.5 microgram) of vitamin B_{12} (11, 12), inhibition of the absorption of larger test doses has rarely been found (13). The retarded absorption presently observed might be due to interference with absorption by some nonspecific material in the IFC (11). However, the intrinsic factor may have been responsible, since the resulting absorption curves were similar to those observed in control subjects given comparable amounts of cyanocobalamin. Moreover, the addition of IFC resulted in each instance in higher hepatic radioactivity. This suggests that more, rather than less, cyanocobalamin was absorbed in spite of the change in the contour of the absorption curves.

Another possibility must be considered to explain the delayed rise of radioactivity in peripheral blood samples in the presence of intrinsic factor. With intrinsic factor there could be early removal of the absorbed vitamin B_{12} by peripheral tissues or organs such as the liver. Simultaneous radioactivity measurements of blood samples from the portal vein would be most helpful to place the cause of the slower rise in plasma radioactivity. Such studies are not available. However, Booth and Mollin (4) found maximum radioactivity in the plasma 8 to 12 hours after dosage while the highest counts over the liver were not obtained until two to six days later. On the other hand, we have obtained a plasma absorption curve in one patient with an effective portacaval shunt. The subject, a 46-year-old man with Laennec's cirrhosis, was studied 17 months after operation. As seen in Figure 2, there was a delay in the peak of radioactivity in the plasma identical to that found in normal subjects. These observations make it unlikely that the liver immediately removes cyanocobalamin from the blood under the influence of intrinsic factor and thereby causes the delay in the rise of radioactivity in the plasma. Moreover, we are not aware of any evidence which indicates

that peripheral tissues selectively remove and temporarily store newly absorbed vitamin B_{12} after oral administration. Therefore, it seems more likely that the intestine is in some way responsible for the slower rise in radioactivity in the plasma in the presence of intrinsic factor. This view is in agreement with that suggested by Booth and Mollin (4).

Tests in control subjects showed that the inhibition of the early rise of radioactivity in the plasma could be overcome by larger doses of vitamin B_{12}. Examination of Figure 5 shows that in one of two control subjects given 100 micrograms and in the subjects tested with 200 micrograms there was considerable plasma radioactivity by four hours, as compared with the results at the 10- and 50-microgram levels. With 300- and 500-microgram doses relatively high plasma radioactivity was found at two and three hours. Then, in contrast to the results obtained in patients with pernicious anemia, the peak was followed by a plateau-like curve. This prolongation was probably due to the superimposition of absorption mediated by intrinsic factor.

The results of these studies lend support to the hypothesis that there are two modes of intestinal absorption of cyanocobalamin: one, mediated by intrinsic factor, is characterized by more gradual and prolonged plasma absorption curves; the other, found with much larger doses of cyanocobalamin, is characterized by early rises in plasma levels of vitamin B_{12}, as indicated by measurements of radioactivity. It has been suggested that this latter mechanism is due to a passive diffusion across the intestinal barrier (9). However, in pernicious anemia the four- to six-hour peak amounts of plasma radioactivity (Figure 3) appeared later than would be expected from a process of simple diffusion. The particular curves can be compared to the plasma absorption curves of iron which are believed to be related to an *active* process of absorption. Oral administration of iron results in peak plasma concentrations two-and-one-half to five hours after test doses (14). Until more details are known it is perhaps best to refer to the two mechanisms of absorption of vitamin B_{12} as the one mediated by intrinsic factor, and the other as absorption independent of this factor.

Recently, it has been estimated that 100 grams of undried beef liver may contain from 60 to 118 micrograms of vitamin B_{12} (15, 16). Figure 3 includes plasma absorption curves with dosages in this general range. Such absorption suggests the possibility that the success of the dietary liver therapy of Minot and Murphy (17) was due to the cyanocobalamin content itself, and not necessarily due to other factors present in the liver which may have promoted hematopoiesis or vitamin B_{12} absorption.

Clinical experience has indicated a wide variation in results in the treatment of pernicious anemia patients with oral doses of cyanocobalamin. Whereas some may show an almost optimal response to daily doses of 15 micrograms (18) and some can be successfully treated with daily oral doses of 50 micrograms (19), failures have been reported with daily doses of as much as 250 micrograms (20–22). This variation is well illustrated in Figure 3 at the 50-microgram dose. Two patients had significant plasma absorption curves and hepatic radioactivity (Table I), while a third patient showed little absorption and only one-half to one-sixth the liver radioactivity of the other two. These findings indicate great individual variation in the absorption gradient in patients with pernicious anemia for doses of vitamin B_{12} above the physiologic range. Such a variation in the absorption gradient is well known also for test doses of a more physiologic magnitude, when a source of intrinsic factor is added (23).

The plasma absorption curves determined by bioassay of vitamin B_{12} after the administration of massive doses of the vitamin have shown peak plasma concentrations 1 to 24 hours after the administration of the tests, and no definite separation between patients with pernicious anemia and control subjects has been claimed (1, 2). This is in contrast to the definitive results obtained when test doses of 50 to 300 micrograms were given. However, with the larger test doses there was less difference between the absorption curves of pernicious anemia patients and normal subjects. This was probably due to the fact that as the test doses were increased, the absorption mechanism independent of intrinsic factor gradually became the more dominant also in the control subjects.

The calculated amount of vitamin B_{12} absorbed was found to range from about 18 micromicrograms per ml. of plasma in a control subject given the 50-microgram test dose to 83 micromicrograms

per ml. of plasma when 500 micrograms were administered. The figures were not too different in the patients with pernicious anemia, except in the patient who absorbed only a minor amount when the 50-microgram test dose was given. Our data are too few to make a more detailed comparison between the amounts calculated to have been absorbed in each group.

Because such small amounts of cyanocobalamin were absorbed it is understandable that bioassay has failed to reveal definite changes in the concentration of vitamin B_{12} in the serum after single oral test doses of less than 500 micrograms (9, 18). Tracer techniques, being more sensitive, have the further advantage that they can determine absorption without measurable changes in the total amount of vitamin B_{12} in the serum.

SUMMARY AND CONCLUSIONS

1. Studies of the rate of entrance of vitamin B_{12} into the plasma have been carried out in eight pernicious anemia patients and eight control subjects after the oral administration of test doses varying from 0.56 to 500 micrograms of Co^{58}- or Co^{60}-labeled vitamin B_{12}.

2. Negligible or no plasma absorption was observed in two patients with pernicious anemia when oral test doses of 0.56 microgram of vitamin B_{12} were administered without intrinsic factor concentrate. When the latter was administered, absorption curves were obtained which were similar to those previously found in control subjects given test doses in the same range. They were characterized by delayed plasma radioactivity appearing after four hours, with a peak concentration at eight hours following dosage.

3. Oral test doses of 50 to 300 micrograms of vitamin B_{12}, given without IFC to patients with pernicious anemia, were followed by differing absorption curves characterized by an early rise in plasma radioactivity and peak concentrations four to six hours after the test. The addition of IFC abolished the early rise in plasma concentration and resulted in a delayed peak concentration at 12 hours. Although IFC delayed the appearance of vitamin B_{12} in the plasma, it enhanced hepatic uptake of radioactivity.

4. Control subjects given test doses of 200 to 500 micrograms of vitamin B_{12} displayed a slightly diphasic absorption into the plasma, with an early rise in the radioactivity. A test dose of 10 micrograms caused a delayed absorption similar to the results observed with the much smaller test doses, while doses of 50 to 100 micrograms resulted in absorptions of an intermediate nature.

5. The present findings lend support to the concept of a dual mechanism of absorption of vitamin B_{12} from the gastrointestinal tract.

ACKNOWLEDGMENT

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PLASMA LIPID INTERRELATIONSHIPS IN EXPERIMENTAL NEPHROSIS [1]

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The injection of rabbit anti-rat kidney serum induces a nephrotic state in rats which closely resembles that occurring in human subjects with the nephrotic syndrome (1). Earlier studies from this laboratory (2–7) indicated that the increase of plasma lipids which occurs in the nephrotic rat's plasma is primarily due to an intravascular "trapping" phenomenon whereby the animal is unable to clear lipid from the plasma with his customary efficiency. It also was found (8, 9) that the profound deficiency of albumin characteristic of the nephrotic rat's plasma appears to be causally responsible for this retention of excess lipid in his plasma. A contributing factor may be the renal loss of heparin or its plasma equivalent, lipoprotein lipase (10). However, the sequential mechanism whereby the deficiency of albumin, and possibly also of lipoprotein lipase in nephrotic plasma, initiates and maintains the increased plasma content of all lipid fractions, remains to be elucidated. This problem has been investigated in the present studies.

METHODS AND RESULTS

Young adult, male rats of the Long-Evans strain (weight, 190 to 225 Gm.) were used in the following studies. Rabbit anti-rat kidney serum (AKS) was prepared by the method of Heymann and Lund (1). Where designated, continuous intravenous infusion was accomplished by means of a special constant infusion apparatus connected to a polyethylene cannula in an inferior lumbar vein, as described previously (8). The studies were performed after an overnight fast, and food was withheld during the experimental intervals.

I. *The serial interrelationships of the plasma lipid changes in rats injected with AKS*

Methods

A. One ml. of pooled AKS was injected into each of 120 rats. At serial intervals during the ensuing 168 hours a separate group of 10 rats was sacrificed and bled from the aorta for determination of plasma albumin (8), total lipids (9), phospholipids (10) and total cholesterol (11). Plasma triglycerides were calculated by difference (subtracting total cholesterol and phospholipids from total lipid). Each rat was bled only once, to avoid the effects of serial sampling. Control values were established in an additional group of 25 normal rats.

B. In order to evaluate further the initial plasma lipid changes induced by AKS injection, an additional series of 20 rats was injected with 1.2 ml. of pooled AKS. Seven of the rats were sacrificed five hours later and the remaining 13 rats eight hours after AKS injection, at which time the rats were bled from the aorta for determination of plasma albumin, total lipids, phospholipids, and total cholesterol.

Results

A. In Figure 1 it can be seen that the injection of AKS in the first series of rats induced a progressive fall of plasma albumin which preceded the rise of plasma lipids. Thus the plasma albumin fell from an initial average of 3.1 Gm. per 100 ml. to an average of 0.5 Gm. per 100 ml. at 24 hours, a level which prevailed throughout the remainder of the experimental interval. There was a parallel rise and later partial fall of plasma phospholipids and total cholesterol. Therefore, for purposes of illustration the concentrations of phospholipids and cholesterol have been combined in Figure 1 in which the plasma triglycerides are represented by the difference between the average concentrations of total lipids and those of the combined cholesterol and phospholipids. It can be seen that, although there was a progressive rise of all three plasma lipid fractions, the rise of plasma triglycerides occurred at a more rapid rate and was of considerably greater magnitude than that of the other lipid fractions. After reaching a peak rise at 96 hours a partial fall of the elevated lipid levels occurred, during which it was observed that the plasma triglycerides fell much more rapidly than did the other lipid fractions.

B. The results of the second series of rats are presented in Figure 2 in which it can be noted that

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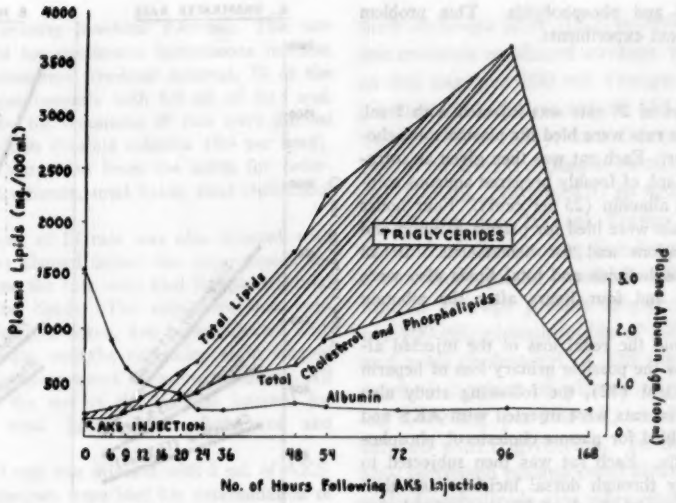
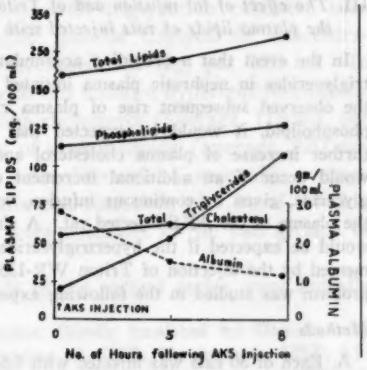


FIG. 1. THE TEMPORAL RISE OF PLASMA LIPIDS IN RATS INJECTED WITH ANTI-KIDNEY SERUM

a progressive fall of plasma albumin and associated rise of plasma lipids again was induced by AKS injection. As shown in Figure 2, triglyceride was the first plasma lipid fraction to rise following AKS injection. Thus, five hours after AKS injection, the plasma triglycerides had risen from an initial average of 20 mg. per 100 ml. to 53 mg. per 100 ml. At the same time no significant change of average plasma cholesterol or phospholipid was observed. Eight hours after AKS injection the plasma triglycerides rose further, to an average of 110 mg. per 100 ml., at which time a slight rise of plasma phospholipid but no change of plasma cholesterol was observed. The range of values and statistical evaluation also are shown in Figure 2.

II. *The fall of plasma lipids in nephrotic rats injected with albumin*

The results of the previous experiment were consistent with the concept (8, 12, 13) that a preceding accumulation of plasma triglycerides is the factor initiating and sustaining the rise of the other plasma lipid fractions. However, if this concept were valid it would be expected that, during the fall of the elevated plasma lipids previously shown to occur in nephrotic rats following injection of albumin (8, 12, 13), the plasma triglycerides would fall at a more rapid rate than would the concentra-



	No. of Hours		
	0	5	8
TOTAL LIPIDS	191	235	303
Range:	(135-225)	(203-262)	(250-380)
S.E. Mean:	±9.0	±7.3	±9.2
TRIGLYCERIDES	20	53	110
Range:	(0-44)	(30-74)	(76-165)
S.E. Mean:	±3.9	±5.0	±7.5
PHOSPHOLIPIDS	114	121	130
Range:	(83-130)	(112-138)	(111-154)
S.E. Mean:	±3.6	±4.0	±3.3
TOTAL CHOLESTEROL	56	63	62
Range:	(40-60)	(46-74)	(44-72)
S.E. Mean:	±2.1	±3.9	±2.0

FIG. 2. THE INITIAL PLASMA LIPID CHANGES IN RATS INJECTED WITH ANTI-KIDNEY SERUM

tions of cholesterol and phospholipids. This problem was studied in the next experiments.

Methods

A. Each of a series of 24 rats was injected with 1 ml. of pooled AKS. The rats were bled for plasma total cholesterol 72 hours later. Each rat was then given an intravenous injection of 3 ml. of freshly prepared aqueous solution of bovine serum albumin (25 per cent). Four hours later the animals again were bled for plasma albumin and cholesterol concentrations and for hematocrits. In 13 instances plasma phospholipids and total lipids also were determined prior to and four hours after the albumin injection.

B. In order to avoid the renal loss of the injected albumin (8), as well as the possible urinary loss of heparin or its plasma equivalent (12), the following study also was performed. Seven rats were injected with AKS and five days later were bled for plasma cholesterol, phospholipids, and total lipids. Each rat was then subjected to bilateral nephrectomy through dorsal incisions and then injected intravenously with 3 ml. of 25 per cent solution of bovine serum albumin. Four hours later the rats were bled for plasma albumin, hematocrits, total lipids, phospholipids and total cholesterol.

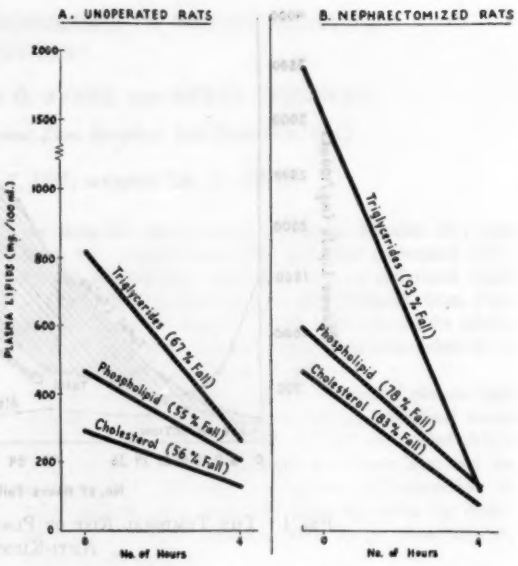


FIG. 3. THE FALL OF PLASMA LIPIDS IN CHRONIC NEPHROTIC RATS INJECTED WITH ALBUMIN

Results

A. The average fall of the plasma lipid fractions induced by albumin injection in the intact rats is shown in Figure 3, A. The plasma triglyceride concentration fell from an initial average of 816 mg. per 100 ml. to 270 mg. per 100 ml., a fall of 67 per cent. The plasma phospholipids exhibited a 55 per cent fall from an initial average of 467 mg. per 100 ml. to 211 mg. per 100 ml., and the plasma total cholesterol a 56 per cent fall from an initial average of 295 mg. per 100 ml. to 129 mg. per 100 ml. At the time of the final bleeding the average plasma albumin concentration was 2.9 Gm. per 100 ml. (range, 1.2 to 4.6) and the average hematocrit was 36 (range, 31 to 39).

B. The second series of rats was studied at a later interval following AKS injection than in the previous group and a considerably more intense hyperlipemia was observed at the time of the first bleeding (Figure 3, B). As the probable result of preventing urinary loss of the injected albumin and possibly also of heparin or of lipoprotein lipase (8, 12), a more marked fall of plasma lipids was observed following injection of albumin than occurred in the preceding experiment. Thus, a 93 per cent average fall of plasma triglyceride occurred, from an initial average of 1,866 mg. per 100 ml. to 124 mg. per 100 ml. As in the first series of rats, a relatively lesser fall of the other plasma lipids was observed. Thus the plasma cholesterol fell an average of 83 per cent, from an initial average of 470 mg. per 100 ml. to 78 mg. per 100 ml., and the phospholipids fell an average of 78 per cent, from an initial average of 598 mg. per 100 ml. to 133 mg. per 100 ml. At the time of the final bleeding the plasma albumin averaged 5.5 Gm. per 100 ml. (range, 5.1 to 5.8) and the average hematocrit was 28 (range, 22 to 32).

III. *The effect of fat infusion and of Triton injection on the plasma lipids of rats injected with AKS*

In the event that a preceding accumulation of plasma triglycerides in nephrotic plasma initiated and sustained the observed subsequent rise of plasma cholesterol and phospholipid, it would be expected that an associated further increase of plasma cholesterol and phospholipid would occur if an additional increment of excess triglyceride, given by continuous infusion, was retained in the plasma of the AKS-injected rat. A similar sequence would be expected if the hypertriglyceridemia was augmented by the injection of Triton WR-1339 (14). This problem was studied in the following experiments.

Methods

A. Each of 30 rats was injected with 0.6 ml. of pooled AKS, an amount which was found in preliminary studies to be inadequate to induce any significant rise of plasma

lipids during the ensuing five-hour interval. The rats then were prepared for continuous intravenous infusion and, during the subsequent five-hour interval, 10 of the rats were infused continuously with 6.0 ml. of fat [2] and, for control purposes, the remaining 20 rats were infused with 6.0 ml. of sodium chloride solution (0.9 per cent). The animals then were bled from the aorta for determination of plasma albumin, total lipids, total cholesterol and phospholipids.

B. A second series of 13 rats was also injected with AKS, but 24 hours elapsed before the experimental interval. At that time the rats were bled for plasma total cholesterol and total lipids. The rats were then continuously infused for five hours, five being injected with 6.0 ml. of triglyceride, and the remaining eight control rats receiving a similar amount of saline solution. All rats were bled at the end of the infusion interval for plasma albumin, total lipids, total cholesterol and phospholipids.

C. A series of 10 rats was injected with 1 ml. of AKS. Fifty hours later the rats were bled for determination of plasma total lipids, phospholipid, and total cholesterol and found to be hyperlipemic. Five of the rats were then injected intravenously with 100 mg. of Triton WR-1339 (contained in 1 ml.), the remaining rats serving as controls. All rats were bled 24 hours later for plasma total lipids, phospholipid, and total cholesterol.

Results

A. The results obtained in the first series of rats are shown in Figure 4, A. In the five hours following injection of the smaller amount of AKS only an occasional saline-infused control rat developed any significant increase of plasma lipids: the average plasma triglycerides was 52 mg. per 100 ml.; average plasma cholesterol, 72 mg. per 100 ml.; and the average plasma phospholipids, 132 mg. per 100 ml. As shown in Figure 4, A, the rats infused with triglyceride exhibited considerably higher plasma triglyceride concentrations, which averaged 635 mg. per 100 ml. In association with this induced hypertriglyceridemia, a rise of plasma cholesterol (average, 115 mg. per 100 ml.) and of phospholipids (average, 220 mg. per 100 ml.) was observed. Comparable average plasma albumin concentrations of 1.4 and 1.5 Gm. per 100 ml. were observed in the two groups of rats.

B. The rats of the second series were initially bled 24 hours after AKS injection, at which time the controls exhibited average plasma total lipids of 445 mg. per 100 ml. (range, 397 to 514) and plasma total cholesterol of 114 mg. per 100 ml. (range, 93 to 131), and the experimental group exhibited average plasma total lipids of 432 mg. per 100 ml. (range, 410 to 481) and cholesterol of 108 mg. per 100 ml. (range, 99 to 120). At the end of the ensuing five-hour infusion interval the saline-injected controls (Figure 4, B) exhibited average plasma triglycerides of 126 mg. per 100 ml., phospholipids of 196 mg. per 100 ml., and total cholesterol of 128 mg. per 100 ml. A marked increase of plasma triglycerides, averaging 2,497 mg. per 100 ml., was induced in the rats infused with triglyceride. In these rats considerably increased plasma concentrations of cholesterol and phospholipids also were observed, respectively averaging 226 mg. per 100 ml. and 364 mg. per 100 ml. Comparable average plasma albumin levels of 0.6 and 0.5 Gm. per 100 ml. were observed in the two groups of rats.

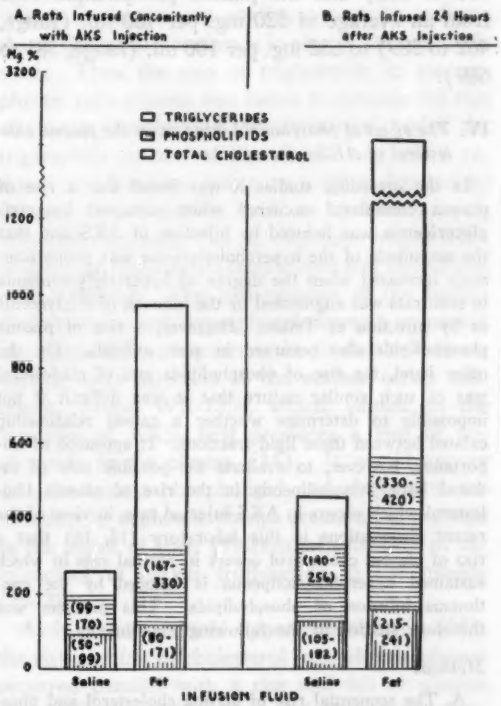


FIG. 4. THE EFFECT OF FAT INFUSION ON PLASMA LIPIDS OF RATS INJECTED WITH AKS

[2] Lipid emulsion (kindly furnished by Don Baxter) composed of sesame oil, USP, 10; dextrose, USP, 4.5; purified soya bean lecithin, 0.65; water, q.s. 100 ml. Lipid is present as finely divided particles of 1 μ or less in diameter.

C. A marked further rise of plasma lipids and cholesterol occurred in the nephrotic rats during the 24-hour interval following injection of Triton. Thus the plasma total lipids of the Triton-injected nephrotic rats rose from an average of 2,280 mg. per 100 ml. (range, 1,560 to 3,400) to 4,713 mg. per 100 ml. (range, 4,349 to 5,180), the plasma triglyceride rose from an average of 1,197 mg. per 100 ml. (range, 747 to 2,113) to 2,610 mg. per 100 ml. (range, 2,467 to 3,034). The plasma cholesterol rose from an average of 393 mg. per 100 ml. (range, 278 to 462) to 805 mg. per 100 ml. (range, 712 to 874) and the plasma phospholipid rose from an average of 690 mg. per 100 ml. (range, 535 to 825) to 1,298 mg. per 100 ml. (range, 1,090 to 1,272). At the same time the average plasma total lipids of the control group rose from an average of 1,547 mg. per 100 ml. (range, 1,287 to 1,713) to 2,120 mg. per 100 ml. (range, 1,591 to 2,490), the plasma cholesterol rose from an average of 411 mg. per 100 ml. (range, 264 to 487) to 518 mg. per 100 ml. (range, 432 to 538) and the plasma phospholipid rose from an average of 520 mg. per 100 ml. (range, 462 to 589) to 632 mg. per 100 ml. (range, 581 to 689).

IV. *The effect of phospholipid infusion on the plasma cholesterol of AKS-injected rats*

In the preceding studies it was found that a rise of plasma cholesterol occurred when sustained hypertriglyceridemia was induced by injection of AKS and that the magnitude of the hypercholesteremia was proportionately increased when the degree of hypertriglyceridemia in such rats was augmented by the infusion of triglyceride or by injection of Triton. However, a rise of plasma phospholipids also occurred in such animals. On the other hand, the rise of phospholipids and of cholesterol was of such similar nature that it was difficult if not impossible to determine whether a causal relationship existed between these lipid fractions. It appeared of importance, however, to evaluate the possible role of induced hyperphospholipemia in the rise of plasma cholesterol which occurs in AKS-injected rats, in view of the recent observations in this laboratory (15, 16) that a rise of plasma cholesterol occurs in normal rats in which sustained hyperphospholipemia is induced by the continuous infusion of phospholipids. This problem was therefore studied in the following experiments.

Methods

A. The sequential rise of plasma cholesterol and phospholipids induced by AKS injection was studied by injecting each of a series of 53 rats with 1 ml. of pooled AKS. Groups of 10 to 11 rats were then bled for plasma total cholesterol and phospholipids at 8, 15, 24, 48 and 96 hours following the injection of AKS.

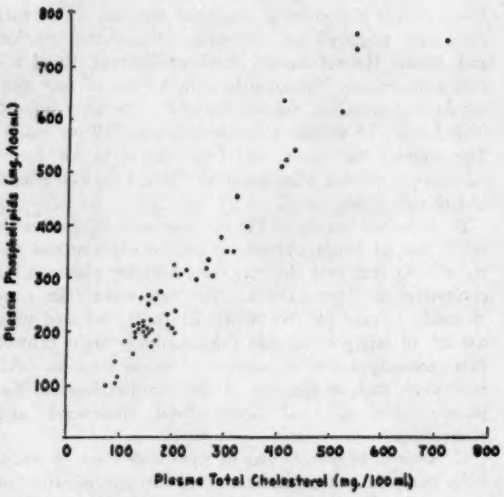


FIG. 5. CORRELATION OF PLASMA PHOSPHOLIPIDS AND TOTAL CHOLESTEROL IN RATS INJECTED WITH ANTI-KIDNEY SERUM

B. The effect of phospholipid infusion in AKS-injected rats was studied in the next experiment. One ml. of pooled AKS was injected into each of 18 rats. During the ensuing 24-hour interval, seven of the rats were continuously infused intravenously with 12 ml. of 2 per cent solution of phospholipids.[3] For control purposes a comparable amount of glucose solution (5 per cent) was infused into six others. The rats were bled for plasma total lipids, total cholesterol and phospholipids at the end of the 24-hour infusion interval.

The remaining five rats were not studied until 20 hours after AKS-injection, at which time they were continuously infused during the ensuing 24-hour interval with 12 ml. of 2 per cent phospholipid solution.[3] The rats were bled for plasma total lipids, total cholesterol and phospholipids prior to and following the infusion interval.

Results

A. A serial parallel rise of the concentrations of plasma cholesterol and phospholipids was induced by AKS injection, as occurred in the first experiment. In Figure 5 it can be seen that the induced rise of plasma total cholesterol in these rats was directly proportional to the rise of plasma phospholipids.

[3] Furnished by Glidden Company as "Soya Lecithin" (RG) brand and dissolved in 5 per cent glucose solution.

B. In the second experiment certain rats were injected with AKS just prior to the continuous 24-hour infusion of phosphatide or glucose. At the end of the infusion interval the rats injected with phosphatide exhibited plasma total lipids averaging 1,012 mg. per 100 ml. (range, 756 to 1,200). There was a marked rise of plasma phospholipids averaging 703 mg. per 100 ml. (range, 522 to 924) and a much smaller rise of cholesterol averaging 213 mg. per 100 ml. (range, 173 to 286). In contrast, the glucose-infused controls exhibited average plasma total lipids of 382 mg. per 100 ml. (range, 350 to 466), average phospholipids of 189 mg. per 100 ml. (range, 148 to 199) and average total cholesterol of 142 mg. per 100 ml. (range, 130 to 148). Thus, no significant increase of plasma triglyceride was induced by the phosphatide infusion.

The remaining rats were injected with AKS 20 hours prior to the infusion of phosphatide. These rats exhibited a marked further rise of plasma phospholipids, from an initial average of 179 mg. per 100 ml. (range, 100 to 236) to 1,038 mg. per 100 ml. (range, 842 to 1,430). There was an associated but relatively much smaller rise of plasma cholesterol from an initial average of 150 mg. per 100 ml. (range, 77 to 208) to 382 mg. per 100 ml. (range, 342 to 396). The plasma total lipids averaged 1,620 mg. per 100 ml. (range, 1,290 to 1,830) at the end of the infusion interval.

DISCUSSION

The results of previous studies from this laboratory have indicated that the increase of lipids in the nephrotic rat is an isolated accumulation of excess lipids which is confined, at least in the early phase, to its plasma (4) and which, although capable of intensification by ingestion of dietary lipid (5), stems primarily from endogenous sources (5). Our studies also indicated that the accumulation of cholesterol in the plasma in this syndrome is not caused by any preceding change in its intestinal absorption (2) or excretion (3), or in the rate of its hepatic synthesis (6). Finally, it was found that both the hyperlipemia and the hypercholesteremia were initiated by the loss of albumin and, conversely, were remedied by the administration of albumin (8, 13) particularly in conjunction with heparin (12).

In the present studies further information was obtained concerning the causal mechanism involved in the hyperlipemia and hypercholesteremia occurring after induction of the nephrotic state. Thus, soon after injection of AKS, a profound fall of plasma albumin was found to occur and, concomitantly with this, the plasma triglyceride, of all the plasma lipid fractions, first begins to rise. It is likely that this retention of excess triglyceride in nephrotic plasma is due to a failure in its hydrolysis consequent to a deficiency of circulating albumin (8) and possibly also of lipoprotein lipase (13), since such lipolysis appears to be a prerequisite for the normal clearance of triglyceride from the plasma (17, 18). The initial rise of triglyceride was followed considerably later by a rise of both plasma phospholipid and cholesterol.

Earlier studies from this laboratory (15, 19) have demonstrated that an accumulation of excess triglyceride in the plasma of the normal rat quickly induces a subsequent rise both of plasma phospholipid and cholesterol. The results obtained in the present studies appear to indicate that a similar mechanism is in play in the nephrotic state. Thus the rise of triglyceride in the nephrotic rat's plasma was found to precede the rise of plasma phospholipid and cholesterol, and if the triglyceride content of the plasma was either reduced by albumin infusion or further elevated by infusion of triglyceride itself, or by Triton injection (14), a prompt fall or elevation, respectively, occurred in the plasma phospholipid and cholesterol concentrations. The pathogenesis of the hypercholesteremia and hyperphospholipemia occurring in nephrosis thus appears to be similar to that occurring after injection of the surface active substance, Triton WR-1339, which, similar to the injection of AKS, first produces a rise of triglyceride in the plasma (14). It would appear that the excess phospholipid and cholesterol accumulate in nephrotic plasma secondarily, perhaps as the result of their preferential solubility in the increment of excess triglyceride in the plasma (19–21).

As in Triton-induced hypercholesteremia (14), the rise and fall of cholesterol in nephrotic plasma occurred parallel with a rise and fall of plasma phospholipids. This suggested that the hyperphospholipemia did not play a major role in the

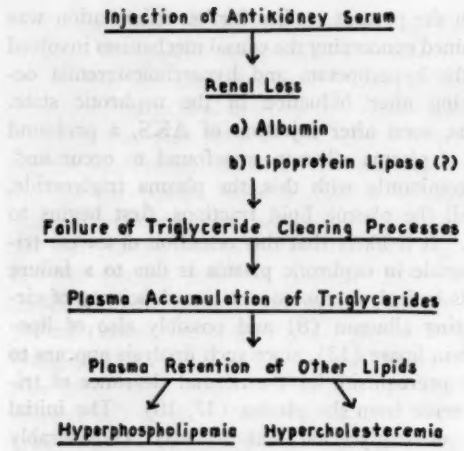


FIG. 6. PATHOGENESIS OF NEPHROTIC HYPERLIPEMIA AND HYPERCHOLESTEREMIA

retention of excess cholesterol in nephrotic plasma, despite the previously demonstrated ability (15, 16) of an induced rise of plasma phosphatide to effect a hypercholesteremia. Moreover, although a further rise of plasma cholesterol did occur in the nephrotic rat, as in the normal rat (15), when phosphatide was infused in an amount sufficient to further raise the plasma phospholipid concentration, the resulting plasma levels were notably disproportionate. Thus, the further rise of plasma cholesterol in such rats was of relatively much less magnitude than the marked induced rise of phospholipids. This could not be ascribed, however, to any inability of such rats to accumulate an additional increment of cholesterol in the plasma at a more rapid rate, since a considerably greater rise of cholesterol was observed in similar nephrotic rats during the infusion of triglyceride or following injection of Triton.

On the basis of the evidence cited above the pathogenesis of the accumulation of excess plasma lipids in experimental nephrosis may be summarized as shown in Figure 6.

SUMMARY

The sequential changes and the interrelationships among the plasma lipid fractions were studied in rats injected with anti-kidney serum, and in nephrotic rats infused with fat or with phosphatide. The results are consistent with the concept that the AKS-induced deficiency of plasma albumin primarily induces an intravascular retention of plasma triglycerides. The accumulation of cholesterol and phospholipid in nephrotic plasma appears to be a passive derangement secondary to the lipid-sequestering properties of the increment of excess triglycerides in such plasma.

ACKNOWLEDGMENT

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MEASUREMENTS OF PULMONARY CAPILLARY BLOOD FLOW AND GAS EXCHANGE THROUGHOUT THE RESPIRATORY CYCLE IN MAN [1]

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Judging from the hemodynamic measurements in the heart and pulmonary circulation as reviewed by Wiggers in 1921 (1) and studied in man by Lauson, Bloomfield, and Cournand in 1946 (2), it seemed entirely possible that the pulmonary capillary blood flow might vary appreciably during the respiratory cycle.

Brecher and Hubay (3) reported respiratory fluctuations of pulmonary artery flow in dogs determined by implanting a bristle flow meter in the pulmonary artery. But if these respiratory variations of blood flow persisted in the pulmonary capillaries in intact man, they could conceivably create a moment-to-moment "unsteady state" which would cause errors in calculation of the respiratory gas exchange and cardiac output.

It has recently been shown that the pulmonary capillary blood flow, as measured by a new and instantaneous method, is pulsatile in time with the heart beat (4). It is the object of this study to measure pulmonary capillary blood flow by the instantaneous method throughout the entire respiratory cycle in the normal intact man.

Previous attempts to measure alveolar capillary gas exchange during the respiratory cycle have been limited to the expiratory phase alone, because expired alveolar gas can be sampled only during expiration. The present method permits measurements to be made of the exchange of gases between alveoli and capillaries during both expiration and inspiration.

METHOD

The rate of absorption of N_2O, a gas which is highly soluble in the bloodstream, was measured by a "manometric respirometer" technique as described previously (4). Figure 1 illustrates the principle of the method. The subject sits entirely inside a body plethysmograph (a large box) which has a sensitive pressure gauge. If nitrous oxide is breathed into the lungs, it is taken up by the blood flowing through the pulmonary capillaries. As N_2O leaves the gas phase, the total gas volume and pressure decrease in the closed box. The blood flow can be calculated by an equation which applies before recirculation, provided the alveolar gas tension and the solubility of the gas in whole blood are known.

In the technique previously described, the measurements of N_2O uptake were made when the breath was held with the glottis open and changes in pressure in the box were due almost entirely to the uptake of N_2O.

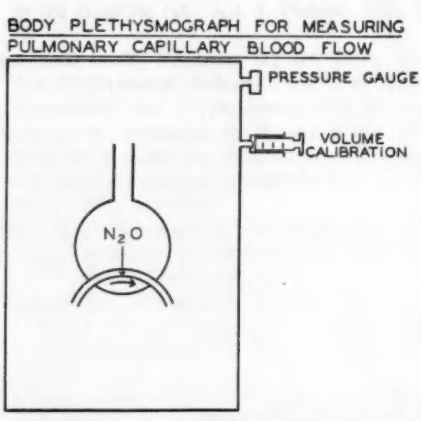

FIG. 1. DIAGRAM ILLUSTRATING THE PRINCIPLE OF MEASUREMENT OF PULMONARY CAPILLARY BLOOD FLOW

During normal respiration the pressure in the box is affected by other factors. First, changes in temperature and saturation of the respired gas cause fluctuations in plethysmographic pressure. These changes were eliminated by rebreathing from bags at body temperature saturated with water vapor. Secondly, changes in alveolar pressure during airflow also produce changes in box pressure (5) but if the airflow is recorded a correction can be applied for the pressure changes due to this cause.

Apparatus. The body plethysmograph used in these investigations has been described previously (4, 5). A double chamber was used; one half was occupied by the

[1] These studies were aided, in part, by a contract between the Office of Naval Research, Department of the Navy, and the University of Pennsylvania, NR 112-323.

[2] This work was done during the tenure of an Established Investigatorship of the American Heart Association.

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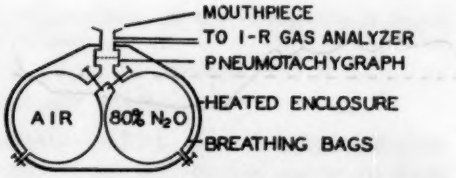

FIG. 2. APPARATUS USED FOR REBREATHING AIR OR 80 PER CENT N_2O SATURATED WITH WATER VAPOR AT 37° C.

subject while the other half at atmospheric pressure merely served to damp sound waves on the reverse side of the manometer. A Lilly capacitance manometer measured the differential pressure between the two chambers and responded with a sensitivity of 0.4 mm. deflection per ml. change of gas volume in the subject's chamber. The natural frequency of this manometer was approximately 200 cps.

Suspended from the roof of the chamber was the heated enclosure containing the gases to be rebreathed (Figure 2).[4] The enclosure consisted of a wire mesh cage encircled by a heating coil. The enclosure was covered by cloth for thermal insulation and by a polyethylene bag to prevent passage of warm air to the outside of the bag as the subject rebreathed. Inside the enclosure were two 4-liter anesthesia bags, one containing air and the other a mixture of 80 per cent N_2O and 20 per cent O_2. The necks of the bags were occluded by clamps and were connected via a 1-inch bore T piece to a small heated flow meter and mouthpiece. The gases put into the bags had been saturated with water vapor by passing them through DeVilbiss nebulizers. The temperature of the heating coil surrounding the enclosure was controlled by means of a variable transformer to maintain the air temperature at 37° C. A 1-mm. bore polyethylene tube led from the mouthpiece of the breathing apparatus to a rapid infrared gas analyzer.[5] This analyzer had a range of zero to 100 per cent N_2O and was located outside the respiratory chamber.

After passing through the analyzer, the gas sample was returned to the chamber by a continuously pumping system through a polyethylene tube. Electrocardiograph leads and an esophageal pressure tubing passed to the outside of the chamber for amplification and continuous recording, by Hathaway oscillograph galvanometers of suitable sensitivity and frequency response, on 6-inch wide photosensitive paper moving at a speed of 1 inch per second. The subject's chamber was vented to the atmosphere at will by a solenoid operated valve. The plethysmograph was calibrated by a small pump which had a to and fro stroke of 30 ml. at a rate of 2 cycles per second.

Procedure. After the subject entered the chamber, it was closed and intermittently vented during a 2-minute warm-up period necessary for the thermal pressure drift to become negligible. Wearing a nose clip, he then breathed approximately half-way out, placed his mouth on the rubber mouthpiece, unclamped the 4-liter bag of air, took a full inspiration from the bag, and then rebreathed at normal rate and depth for a period of 20 seconds. The clamp was replaced on the neck of the air bag. After 2 minutes of recovery, he again breathed half-way out, put his mouth on the mouthpiece, unclamped the other gas bag which contained 4 liters of a mixture of 80 per cent N_2O and 20 per cent O_2, took a deep breath of the mixture and then rebreathed at a normal rate and depth for a 20-second period. At the end of that time, he removed his mouth from the mouthpiece. Continuous recordings were made during the control period on air and during the experimental period on nitrous oxide. The pressure gauges and nitrous oxide meter were calibrated. This procedure was carried out during normal quiet breathing and repeated with the subject breathing through added resistance.

A breathing resistance consisting of glass wool packed into a 1-inch breathing tube was interposed between the mouth of the subject and the rebreathing apparatus. The value of this resistance, measured by pressure-flow tracings at 0.5 L. per second, was 10 cm. H_2O per L. per second, which is in the range of moderate to marked obstruction to breathing. The usual procedures rebreathing air and N_2O were carried out, but in addition esophageal pressure fluctuations were recorded from an air-filled balloon 10 cm. long placed in the lower third of the esophagus, to show that fluctuations of pleural pressure were taking place, as seen in Figure 3, and to rule out changes of resistance which might otherwise affect the correction for gas compression during breathing.

The usual respiratory cycle included approximately three heart beats during inspiration and three beats during expiration. Additional experiments were performed to determine the effect upon blood flow of slow breathing, at the rate of approximately five heart beats during inspiration and five during expiration, *i.e.*, about seven respirations per minute.

During ordinary breathing, there was but little variation of heart rate throughout the respiratory cycle. However, during slow breathing there was considerable sinus arrhythmia. Atropine, 1.2 mg., was administered subcutaneously to two subjects to diminish the sinus arrhythmia, in an attempt to separate the mechanical factors from the vagal factors which might affect the pulmonary capillary blood flow.

Calculation. The first respiratory cycle rebreathing N_2O was neglected, but the next few cycles (Figure 3) were measured provided they were "on scale" and prior to recirculation. As many as five consecutive cycles were sometimes measured.

The type of record obtained is shown in Figure 3.

[4] Previous attempts to heat and saturate the entire air inside of the plethysmograph had resulted in discomfort of the subject. However, in the present arrangement the air surrounding the body of the subject was not heated.

[5] Liston-Becker model 16. Case flushed with 100 per cent CO_2 to discriminate against the latter. Lag (Figure 3) was about 0.2 second.

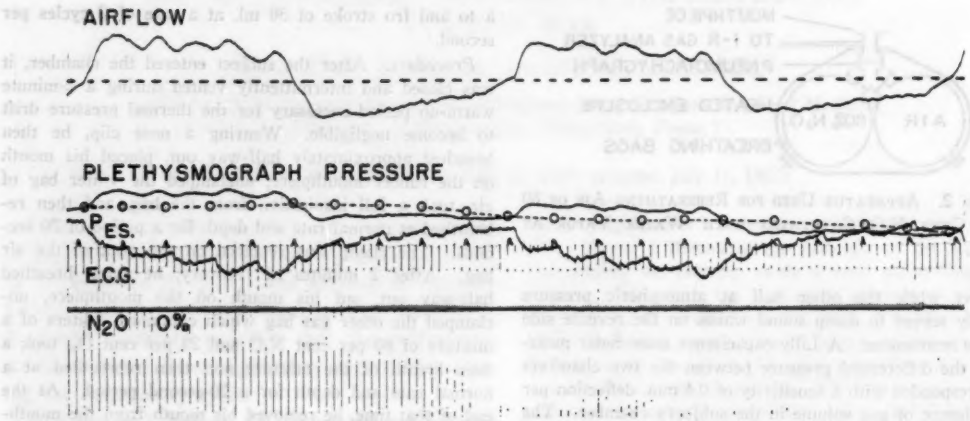

FIG. 3A. SIMULTANEOUS RECORDINGS OF PNEUMOTACHYGRAM, PLETHYSMOGRAPHIC PRESSURE, ESOPHAGEAL PRESSURE AND ELECTROCARDIOGRAM, WITH 0 PER CENT N_2O IN THE MOUTHPIECE WHILE THE SUBJECT WAS BREATHING AIR DURING THE CONTROL PERIOD, PRIOR TO BREATHING NITROUS OXIDE (FIGURE 3B)

Calibration factors and paper speed are same as for Figure 3B. The purpose of the record is to show the magnitude of the correction factors discussed in the section on calculations, particularly the relationship between plethysmographic pressure and the rate of airflow at the mouth.

During the control period on air there were respiratory fluctuations of the plethysmographic pressure proportional to the rate of airflow at the mouth of the subject. With the low rates of airflow obtained during normal breathing (usually less than 0.5 L. per second) this relationship between recorded airflow and plethysmographic pressure was linear and could be measured either from the tracing obtained during normal breathing or during panting at the end of the record. The relationship between deflection recorded by the flow meter and plethysmographic pressure was approximately the same whether breathing air or the foreign gas mixture (approximately 50 per cent N_2O).

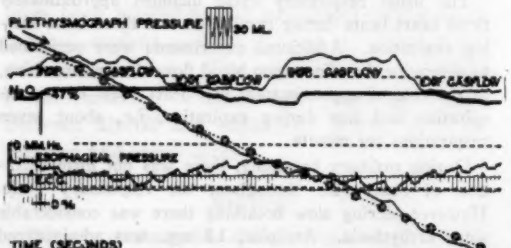

FIG. 3B. SIMULTANEOUS RECORDINGS OF PLETHYSMOGRAPHIC PRESSURE, PNEUMOTACHYGRAM, N_2O PER CENT IN EXPIRED AIR, ESOPHAGEAL PRESSURE AND ELECTROCARDIOGRAM

The record reads from left to right. The R waves of the ECG have been touched up. Inset is calibration of plethysmographic pressure. This subject had atropine. Dotted line through circles represents plethysmographic pressure change attributable to gas absorption, a correction having been made for resistance to airflow.

The plethysmographic pressure during the rebreathing of N_2O was read and the correction subtracted proportional to the rate of airflow. A second correction was applied for the trend of the respiratory exchange difference for O_2 and CO_2, this trend having been determined during the period rebreathing air. In order to avoid the effect of the changes in blood flow which occur during the cardiac cycle (4) the corrections were made at the same phase in each cardiac cycle (the R wave of the ECG).

The blood flow during a given interval of time is given by the following equation which is an application of the Fick principle for bloodflow (4):

$$\dot{Q} = \frac{\Delta P \cdot 60}{470 \cdot F_{A \cdot N_2O} \cdot P_{cal} \cdot \Delta t}$$

where ΔP is the fall in plethysmographic pressure during an interval of time Δt; 470 ml. per L. is the solubility coefficient for N_2O in blood at 37° C.; F_{AN_2O} is the mean concentration[6] of N_2O in the expired alveolar air; P_{cal} is the change in pressure in the plethysmograph for 1 ml.

[6] The mean of expired alveolar N_2O was measured at about mid-expiration. Systematic fluctuations of a few per cent N_2O from the mean (as in Figure 3) were observed during the breathing cycle. Like O_2 and CO_2 (6), the mean during inspiration is probably very nearly the same as the mean N_2O during expiration. The rate of change of alveolar concentration is about one-half per cent per second, (4), which introduces a slight systematic error in end-inspiratory and end-expiratory bloodflow. No attempt was made to correct the calculated bloodflow for respiratory fluctuations of N_2O concentration.

change in gas volume; and $\dot{Q}$ is the rate of blood flow in L. per minute.

RESULTS

Normal quiet breathing

Pulmonary capillary blood flow during inspiratory air flow, expiratory air flow, and for the whole respiratory cycle is tabulated as measured on five normal subjects (Table I). Each subject was measured on two different occasions. Beat-by-beat gas absorption throughout the respiratory cycle was plotted. The percentage deviations from mean blood flow of inspiratory blood flow and of expiratory blood flow are tabulated for individual subjects and averaged for the group of subjects. The results may be summarized as follows: for the group of subjects, the deviation of inspiratory blood flow from the mean blood flow was + 1.8 per cent; S.D., 7.6 per cent; S.E., 2.4 per cent. The deviation of expiratory blood flow from the mean blood flow was − 1.0 per cent; S.D., 7.7 per cent; S.E., 2.4 per cent. By the "t" test, the difference from mean blood flow and the difference between inspiratory and expiratory blood flow are not significant.

Breathing through added resistance

Similar measurements while breathing through added resistance are listed in Table II. No significant fluctuation was found.

Slow breathing

In subject A. B. DB., experiment No. 4, the mean blood flow was 6 L. per minute, and the heart rate was 74 per minute during slow breathing. There was an increase in blood flow to 7 L. per minute toward the end of inspiratory gas flow and decrease toward the end of expiratory gas flow to 5 L. per minute. Cardiac acceleration to 86 also occurred toward end inspiration, and deceleration to 62 toward end expiration.

TABLE I

Pulmonary capillary blood flow during normal quiet respiration

Subject	Subject's expt.	Mean $\dot{Q}$	Insp. $\dot{Q}$	Change	Exp. $\dot{Q}$	Change
	No.	*L./min.*	*L./min.*	*%*	*L./min.*	*%*
R. M.	1	4.7	4.2	−11	5.2	+11
	2	5.7	6.1	+ 7	5.4	− 5
A. B. DB.	1	3.5	3.5	0	3.3	− 6
	2	4.2	4.1	− 2	4.3	+ 5
J. S.	1	7.0	7.8	+11	6.6	− 6
	2	8.2	8.8	+ 7	7.6	− 7
G. B.	1	7.8	7.9	+ 1	7.5	− 4
	2	5.4	5.8	+ 7	5.1	− 6
P. C.	1	6.6	7.1	+ 7	6.3	− 5
	2	5.4	4.9	− 9	6.1	+13
	Mean	5.9		+1.8		−1.0
	S.D.	1.5		7.6		7.7
	S.E.	0.5		2.4		2.4

TABLE II

Pulmonary capillary blood flow while breathing against added resistance

Subject	Subject's expt.	Mean $\dot{Q}$	Insp. $\dot{Q}$	Exp. $\dot{Q}$
	No.	*L./min.*	*L./min.*	*L./min.*
A. B. DB.	3	4.1	4.06	4.3
R. M.	3	6.7	6.9	6.4

Atropine

Of the two subjects given atropine, one performed at a normal respiratory rate and exhibited very little fluctuation of blood flow during the respiratory cycle (Figure 3) whereas the other subject, A. B. DB., breathed slowly and showed fluctuations of blood flow similar to slow breathing without atropine (see above) except that sinus arrhythmia disappeared. These few measurements seem to indicate that further studies of the effects of different breathing patterns, drugs, and disease may be of interest.

DISCUSSION

The evidence presented above leads directly to the conclusion that the exchange of inert, soluble gas between the alveoli and capillaries continues to occur evenly during both inspiration and expiration. Furthermore, it is apparent that pulmonary capillary blood flow does not vary, or varies within narrow limits, throughout the respiratory cycle in normal man.

During the "direct Fick" determination of blood flow it is generally assumed that the respiratory fluctuations of blood flow and alveolar gas tensions do not cause any appreciable error in the measurement of blood flow. The above data show that this is a safe assumption for the lung capillaries, but this does not rule out errors due to fluctuations of blood flow at other sites of blood sampling.

It has been calculated, assuming constant blood flow throughout the respiratory cycle, that there are respiratory fluctuations of alveolar P_{CO_2} and

P_{O_2} amounting to a few mm. Hg of partial pressure (6). The assumption concerning blood flow has now been tested and shown to be reasonably accurate for a normal rate of breathing. However, slow, deep breathing may cause a variation of blood flow such as to slightly diminish the predicted respiratory excursions of alveolar gas tension.

In the introductory paragraphs it was mentioned that certain hemodynamic measurements tended to indicate the possibility of respiratory variations of pulmonary blood flow and vascular resistance. We have not made simultaneous determinations of pulmonary vascular pressure in our subjects, and direct measurements of blood velocity in the great vessels have not been attempted under similar experimental conditions in humans. Knowledge of the mechanical properties of the pulmonary arteries, capillaries and veins is not yet sufficient to allow interpretation of pressure and capillary blood flow in terms of resistance to flow even had they been measured simultaneously, because pressure and flow are pulsatile and not exactly simultaneous with each other. Therefore the following part of the discussion, which is a comparison of our results with the results of others, must be considered as speculative until further investigation of the problems.

It has been shown that the effective pulmonary arterial pressure (*i.e.*, in relation to intrapleural pressure) is raised during inspiration (2, 7, 8), whereas the effective left auricular pressure measured by others (as discussed in Reference 2) shows little change so that pressure difference between these vessels is therefore augmented during inspiration. The rise in effective pulmonary arterial pressure has been supposed by some (8) to be due entirely to the increased output of the right heart during inspiration, since it still occurs when sympathetic nerve action is blocked by tetraethyl ammonium bromide. An increase in the cardiac output of normal subjects, or increase in blood flow through one lung due to clamping the other pulmonary artery, is accompanied by either no rise or only a slight rise in pulmonary arterial pressure. For this reason Brecher and Hubay (3) considered that the small increase in cardiac output which occurred during inspiration was insufficient to account for the rise in pulmonary arterial pressure, and that some increase in pulmonary vascular resistance must be postulated. This increased resistance is probably not due to vasomotor activity (9) but could be due to mechanical changes in the lung vessels during inflation.

Daly (10) found that inflation of the lungs decreased vascular resistance but most workers have found that in the lungs, either isolated or *in situ*, when the vascular pressure is maintained constant in relation to intrapleural pressure, the vascular resistance increases on inflation (7, 11, 12). Direct observation of the lung capillaries in the rat (13) shows that during inspiration the capillaries become flatter and the blood flow in them usually diminishes, probably indicating increased vascular resistance. During normal inspiration the increased output of the right heart must be accommodated by distension of the pulmonary arteries.

During inspiration there is a fall in systemic blood pressure, even when this is measured relative to intrapleural pressure (2, 7, 12, 14–18), and oncometer and cinematographic studies of the heart (16) have shown that this is associated with a diminished left ventricular output and left ventricular diastolic volume. A diminished left atrial size has also been reported during inspiration (14), as would be expected if the diminished left ventricular output is due to decreased filling. Since the blood flow into the capillaries is not diminished during inspiration there must be an increase of blood volume in either the pulmonary capillaries or pulmonary veins. The site of this increase in capacity has not been determined and the evidence is conflicting. Direct observations of the lung capillaries (13) show that they are flattened during inspiration and their volume does not appear to increase. Heinbecker (12), in perfusion studies in which lungs were inflated with positive pressure, also concluded that the capacity of the capillaries was decreased on inspiration. Some workers (17, 19) have found an increased blood volume in the lungs during inspiration, but the experiments were carried out under conditions such that there was an increased pressure across the walls of the pulmonary veins during inspiration and dilatation of these may have accounted for the increased capacity. In the intact chest there is no other reason why the increased negative intrathoracic pressure should dilate the part of the pulmonary veins outside the lung parenchyma.

In summary, it may be said that the increased

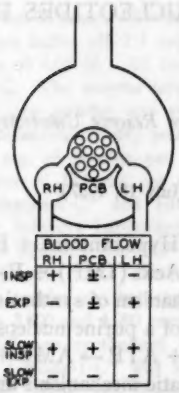

FIG. 4. SCHEMATIC REPRESENTATION OF THE BLOOD FLOW THROUGH THE DIFFERENT BLOOD COMPARTMENTS IN THE THORAX DURING INSPIRATION AND EXPIRATION AT NORMAL AND SLOW RATES

RH = right heart (including pulmonary arterial tree); PCB = pulmonary capillary bed; LH = left heart (including pulmonary veins). The change of blood flow is indicated by + (increase), − (decrease), and ± (no change).

pulmonary artery pressure which occurs during inspiration is probably due partly to an increased output of the right side of the heart and partly to an increased resistance to pulmonary blood flow; as a result, the flow into the capillaries is constant. The reduction of the output of the left heart which occurs a beat or two later can only be explained on the basis of an increased capacity of the pulmonary capillaries or veins but the exact location of this increased capacity and its mechanism are not understood.

When the breathing is slower and deeper it is probable that the increased cardiac output, due to an increased venous return, is greater than can be accommodated by distension of the pulmonary arterial system and the balance between cardiac output and pulmonary vascular resistance is upset, resulting in an increase in pulmonary capillary blood flow towards the end of inspiration. The increased cardiac output is due mainly to increase in stroke volume since the effect is not abolished when the sinus arrhythmia is prevented with atropine. Figure 4 is a schematic diagram which attempts to summarize the findings reported in this paper, and their relationship to the work discussed in the preceding paragraphs.

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MECHANISMS OF SYNTHESIS OF PURINE NUCLEOTIDES IN HEART MUSCLE EXTRACTS[1]

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The key role of ATP, a purine nucleotide, in the conversion of chemical energy into mechanical work by myocardial tissue is well established (1, 2). The requirement for purine nucleotides has also been demonstrated in the multiple synthetic reactions which maintain all animal cells in the steady state. Since the question immediately arises whether the purine nucleotides are themselves in a steady state, in which their rates of synthesis equal their rates of degradation, it seems reasonable to investigate first what mechanisms of synthesis and degradation may be operative.

At present, there are three known pathways for the synthesis of purine nucleotides. The first is the synthesis *de novo* of the purine ring from small molecular weight precursors, such as glycine, formate, CO_2, glutamine and aspartic acid (3). The initial steps in this synthesis (4, 5) are as follows:

1. Ribose-5-phosphate + ATP[3] → 5-Phosphoribosylpyrophosphate (PRPP) + AMP.
2. PRPP + glutamine → 5-phosphoribosylamine (PRA) + glutamic acid.
3. PRA + glycine + ATP → glycinamide ribotide + ADP + Pi.

Glycinamide ribotide is converted to formyl glycinamide ribotide and then through a series of steps to adenylic and guanylic acids (6).

The second pathway available for the synthesis of nucleotides is via the condensation of a free purine base with PRPP (7):

4. Adenine or Hypoxanthine + PRPP → AMP or Inosinic Acid (IMP) + P–P.

The third mechanism of synthesis is through the phosphorylation of a purine nucleoside (8, 9):

5. Adenosine + ATP → AMP + ADP.

Several enzymatic mechanisms are known which result in the degradation of purine nucleotides and nucleosides. The deamination of adenylic acid is well known (10):

6. AMP → IMP + NH_3.

Non-specific phosphatases (11) as well as specific 5′-nucleotidases (12) have been described which result in dephosphorylation:

7. AMP → Adenosine + Phosphate (Pi).

Adenosine can be converted by the enzyme, nucleoside phosphorylase (13), to the free base and a sugar derivative:

8. Adenosine or Inosine+ Pi → Adenine or Hypoxanthine + Ribose-1-Phosphate.

Finally, adenosine may be converted to inosine by an enzyme adenosine deaminase (14):

9. Adenosine → Inosine + NH_3.

The main purpose of the experiments reported in this paper is to indicate which of the enzymes that catalyze the synthesis of purine nucleotides are present in heart muscle extracts. Observations on some of the degradative steps are also presented.

EXPERIMENTAL

Enzyme preparation. Three fresh pig hearts were maintained at 0° C. immediately after removal for one-half hour. After the fat was removed, they were ground together in a refrigerated electric meat grinder. Three hundred Gm. of ground meat was then suspended in 600 ml. of 0.05 M potassium phosphate buffer pH 7.4 and homogenized for 30 seconds in a Waring blendor with a Variac setting of 60 volts. The fluid portion of the homogenate was squeezed through two layers of cheese cloth and centrifuged in a Sorvall Centrifuge (Type SS-1) powered by 60 volts for 15 minutes. A portion of the supernatant solution was kept frozen at −13° C. and the remainder was dialyzed against 20 liters

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[3] Abbreviations are as follows: ATP, adenosinetriphosphate; ADP, adenosinediphosphate; AMP, adenosinemonophosphate; IMP, inosinic acid; PRPP, 5-phosphoribosylpyrophosphate; PRA, 5-phosphoribosylamine; R-5-P, ribose-5-phosphate; Pi, inorganic phosphate; P-P, pyrophosphate; NH_3, ammonia; and PGA, 3-phosphoglyceric acid.

of distilled water for 18 hours. To the dialyzed enzyme, potassium phosphate buffer pH 7.4 was added to make a final concentration of 0.05 M. All operations were carried out at +2° C. The enzyme solution was then lyophilized and the dry powder was stored at −13° C. Prior to each experiment, the dry powder was dissolved in water. Fifty mg. of powder per ml. resulted in a protein concentration of 28.5 mg. per ml.

Substrates. Adenine-8-C^{14} and adenosine-8-C^{14} were obtained from the Schwarz Laboratories. Adenine-8-C^{14} was deaminated to hypoxanthine-8-C^{14} by a modification of the method of Davoll (15). These purine derivatives were diluted with carrier so that the final specific activity was between 3,000 and 4,000 counts per minute per micromole. AMP[28] was synthesized by the method of Eggleston (16). Glycine-1-C^{14} was synthesized by the method of Sakami, Evans, and Gurin (17) and had a specific activity of 21,500 counts per minute per micromole. PRPP was prepared enzymatically by a slight modification of the procedure of Kornberg, Lieberman, and Simms (18). ATP was purchased from Pabst, Inc., and potassium phosphoglyceric acid (PGA) was prepared from the barium salt as described previously (19).

Methods. All reaction mixtures were incubated at 37° C. in air. For determination of the amount of glycine-1-C^{14} incorporated into the glycinamide ribotides and other compounds, the reaction filtrate was treated with ninhydrin to decarboxylate the glycine-1-C^{14}, and the remaining radioactivity was counted (19). The column chromatographic isolation of the glycinamide ribotides has been described (20). For the paper chromatographic separation of other compounds, the following solvent systems were employed: a) butanol saturated with water, 15 N NH_4OH (100:1); b) n-propanol, 15 N NH_4OH, water (60:30:10); c) isopropanol, concentrated HCl, H_2O (170:60:20). The amount of nucleotide synthesized from radioactive adenine, hypoxanthine or adenosine was determined by the application of a 0.2 ml. aliquot of the reaction filtrate to a 5-cm. starting line on Whatman No. 1 paper. The filtrate was chromatographed by the descending method using solvent A, which resulted in movement of nucleosides and free bases off the starting line, but no movement of nucleotides. It was necessary to allow the solvent to run off the paper for an estimated front distance of 45 cm. in order to get good separation of some of the nucleosides from the nucleotides. Solvent B was also employed in some experiments as a check on the isolation of nucleotides. It was possible with solvent B to separate the nucleotides into one band containing adenylic acid and another containing inosinic acid, ADP and ATP. After acid hydrolysis of the material from the second band, and rechromatography of the bases in solvent system A, the radioactivity of the inosinic acid and a mixture of ADP and ATP could be estimated. Paper electrophoretic separations of nucleotides were done with 0.02 M sodium citrate buffer, pH 3.5. Radioactive compounds were eluted from the paper by soaking overnight in 3 ml. of 0.1 N HCl. Aliquots were dried on glass planchets for radioactivity determinations by an end-window counter. Other aliquots were used for the determination of spectra and the estimation of the amount of material recovered. An extinction coefficient for AMP and IMP of 14.8 was employed. The results reported in the tables represent single experiments. Preliminary experiments with the different precursors yielded comparable results.

RESULTS

Synthesis of nucleotides by the de novo pathway

Incubation of glycine-1-C^{14} with the fresh extract (Table I) and no substrate additions resulted in 0.003 micromole of glycine fixed in one hour. Addition of ribose-5-phosphate (R-5-P), ATP and glutamine increased fixation to 0.006 micromole. Substitution of 5-phosphoribosylpyrophosphate (PRPP) for R-5-P increased fixation by slightly more than three-fold while substitution of 5-phosphoribosylamine (PRA) for PRPP and glutamine again increased fixation by approximately three-fold to 0.066 micromole. It should be emphasized that glycine fixation represents incorporation into any compound in which the glycine is not decarboxylated by ninhydrin. An attempt at identification of the radioactive products by ion exchange chromatography and paper chromatography was unsuccessful. No radioactivity was found at the points of elution of glycinamide ribotide or its formyl derivative. Thus the glycine incorporation, stimulated by purine precursors, could have been in other intermediates and the 0.006 micromole of glycine fixed when R-5-P and glutamine were added probably repre-

TABLE I

Fixation of glycine-1-C^{14} in the presence of purine nucleotide precursors *

Additions	Glycine fixed
	micromoles
None	0.003
R-5-P + glutamine	0.006
PRPP + glutamine	0.020
PRA	0.066

* Each reaction vessel contained: ATP, 0.8 micromole; PGA, 7 micromoles; glycine-1-C^{14}, 5 micromoles; $MgCl_2$, 5 micromoles; Tris buffer pH 7.0, 20 micromoles; undialyzed pig heart extract, 4.1 mg. protein. Additions were: R-5-P, 5 micromoles; glutamine, 5 micromoles; PRPP, 1.2 micromoles; PRA, approximately 1 micromole. The final volume was 1 ml. and the reaction mixture was incubated for 1 hour at 37° C.

TABLE II

Synthesis of nucleotides from C^{14} adenine and PRPP *

Experiment	Conditions	Time	C^{14} adenine incorporated	U.V. absorbing material
		min.	*micromoles*	*micromoles*
1	Undialyzed Extract	60	0.11	
	Undialyzed Extract + PRPP	60	0.17	
2	Undialyzed Extract + PRPP	60	0.13	
	Dialyzed Extract + PRPP	60	0.20	
3	Dialyzed Extract + PRPP	15	0.035	0.182
	Dialyzed Extract + PRPP	30	0.074	0.240
	Dialyzed Extract + PRPP	45	0.112	0.288
	Dialyzed Extract + PRPP	60	0.164	0.360

* Each reaction vessel contained: adenine-8-C^{14}, 1 micromole; $MgCl_2$, 2.5 micromoles; Tris buffer pH 7.0, 20 micromoles; dialyzed heart muscle extract, 5.7 mg.; PRPP addition, 0.75 micromole. The final volume was 0.50 ml. and vessels were incubated at 37° C.

sents a maximum value for *de novo* synthesis under these conditions.

Synthesis of nucleotides by the reaction of purine bases with PRPP

All of the experimental results presented in Tables II and III indicate that an enzyme is present in heart muscle which catalyzes the synthesis of purine nucleotides from PRPP and adenine or hypoxanthine. In experiment 1, Table II, in undialyzed extracts, addition of PRPP increased synthesis by 55 per cent. Dialyzed extract (experiment 2) appeared to be more active than undialyzed extract. Synthesis proceeded in a linear fashion (experiment 3) under these conditions for at least one hour. That the C^{14} incorporation represents net synthesis rather than exchange is suggested by the net increase with time of nucleotide material as determined by optical density (O.D.) at 260 mμ. This increase probably represents synthesis of purine nucleotides, mainly from the C^{14} adenine-PRPP reaction, and secondarily from some unlabeled precursor.

When ribose-5-phosphate and ATP were substituted for PRPP, synthesis of purine nucleotides was effective (Table III, experiment 1). There appeared to be an optimal level of ATP as was indicated by Kornberg, Lieberman, and Simms using a pigeon liver enzyme (18). Not only is adenine utilized in the synthetic reaction, but also hypoxanthine can be incorporated (Table III, experiment 2).

The nucleotide products recovered from the enzymatic reaction between adenine and PRPP were identified as adenylic acid and inosinic acid. In Table IV, it is clear from the total amounts recovered that the main product was adenylic acid. Furthermore the higher specific activity of adenylic acid suggests that it is the precursor of inosinic acid. Adenylic acid was identified chromatographically by its movement in solvent system B, by its movement on paper electrophoresis, and by hydrolysis and identification of the free base as adenine in solvent system A. Inosinic acid, the only nucleotide product of the reaction between hypoxanthine and PRPP, was identified by paper chromatography in solvent system B, by spectra, and by movement in paper electrophoresis at pH 3.5.

TABLE III

Synthesis of nucleotides from C^{14} adenine, C^{14} hypoxanthine, PRPP and R-5-P *

Experiment	Conditions	C^{14} purine incorporated
		micromoles
1	Adenine + R-5-P + 0.4 micromole ATP	0.047
	Adenine + R-5-P + 0.8 micromole ATP	0.023
	Adenine + R-5-P + 2.0 micromoles ATP	0.020
	Adenine + PRPP	0.160
2	Adenine + PRPP	0.160
	Hypoxanthine + PRPP	0.130

* Each reaction vessel contained $MgCl_2$, 2.5 micromoles; Tris buffer pH 7.0, 20 micromoles; and dialyzed heart muscle extract, 5.7 mg. Additions were R-5-P, 2.5 micromoles; ATP as indicated in table; PGA, 14 micromoles; PRPP, 0.75 micromole; adenine-8-C^{14}, 0.98 micromole; hypoxanthine-8-C^{14}, 0.70 micromole in a final volume of 0.50 ml.

Synthesis of nucleotides from adenosine and ATP

It is evident from the experimental results presented in Table V, that an adenosine kinase exists in heart muscle. Experiment 1 shows the synthesis of nucleotide from C^{14} adenosine as a function of time. Under these conditions, the synthesis is not linear. This is probably not due to insufficient ATP, as an ATP regenerating system (PGA and the required enzymes) is present, but is more likely due to levels of adenosine insufficient to saturate the enzyme because of the strong competing reaction catalyzed by adenosine

deaminase. If one assumes the enzyme is saturated for the first 15 minutes, then the minimum rate of synthesis per hour is 0.37 micromole. Results of experiment 2 indicate that ATP is required for synthesis of nucleotides from C^{14} adenosine. Isolation of C^{14} inosine and hypoxanthine (experiment 2) after a one-hour incubation is strong evidence for the presence of adenosine deaminase and nucleoside phosphorylase. No C^{14}-adenosine was recovered. The total C^{14} recoveries in this experiment were 87 and 90 per cent. In experiment 2 the amount of nucleotide synthesis is decreased because one-half the amount of C^{14} substrate was employed. Results of experiment 3 indicate that different levels of ATP are not critical in the reaction. Again radioactive inosine and hypoxanthine were recovered as well as 0.26 micromole of an unidentified fraction. The total C^{14} recovery in this experiment was 75 per cent. The synthesis of C^{14}-labeled nucleotides from C^{14} adenosine could go via inosine and hypoxanthine with condensation of the latter with PRPP, as indicated in Table III. Since, in experiment 4, a pool of non-radioactive hypoxanthine did not decrease the C^{14} incorporated into nucleotides, it may be concluded that hypoxanthine is not an intermediate in the conversion of adenosine to a nucleotide. Non-radioactive inosine and adenine gave similar results.

The radioactive products of the reaction between C^{14}-adenosine and ATP were identified by paper chromatography of the nucleotides in solvent B, and of the free bases of the nucleotides in solvent A, and also by paper electrophoresis of the nucleotides. After a one-hour incubation, approximately 10 per cent of the total C^{14} recovered was adenylic acid, 10 per cent was inosinic acid, and 80 per cent was a mixture of ADP and ATP.

TABLE IV

Nucleotide products of the reaction between PRPP and C^{14} adenine *

	Nucleotides	
	Inosinic acid	Adenylic acid
R_F in solvent B	0.15	0.22
Micromoles C^{14} incorporated	0.06	0.14
Specific activity in counts/min./micromole	940	3,260
Per cent of specific activity of original adenine	24	83

* Products were recovered from an experiment similar to that in Table III, experiment 2.

Dephosphorylation of adenylic acid

A final experiment was done to demonstrate that an enzyme is present in heart muscle extract which can liberate phosphate from adenylic acid. Six and three-tenths micromoles of adenosine-5-phosphate labeled with P^{32} were incubated with 5 micromoles of $MgCl_2$, 20 micromoles of Tris buffer pH 7.0, and 5.7 mg. of dialyzed enzyme in a volume of 0.8 ml. for 60 minutes. The TCA reaction filtrates of the zero time control and the incubation mixture were then chromatographed in

TABLE V

Synthesis of nucleotides from C^{14} adenosine and ATP *

Experiment	Conditions	Time	C^{14}-labeled compound recovered		
			Nucleotide	Inosine	Hypoxanthine
		minutes	*micromoles*		
1	Adenosine (3.9)† + ATP (2.0)	15	0.092		
	Adenosine (3.9) + ATP (2.0)	30	0.104		
	Adenosine (3.9) + ATP (2.0)	60	0.134		
2	Adenosine (1.8)	60	0	0.99	0.57
	Adenosine (1.8) + ATP (0.8)	60	0.048	1.08	0.51
3	Adenosine (3.9) + ATP (0.8)	60	0.12		
	Adenosine (3.9) + ATP (2.0)	60	0.15	1.90	0.62
4	Adenosine (1.8) + ATP (2.0)	60	0.067		
	Adenosine (1.8) + ATP (2.0) + hypoxanthine (5)	60	0.075		
	Adenosine (1.8) + ATP (2.0) + inosine (5)	60	0.067		
	Adenosine (1.8) + ATP (2.0) + adenine (5)	60	0.056		

* The reaction vessels contained additions as indicated, plus $MgCl_2$, 2.5 micromoles; Tris buffer pH 7.0, 20 micromoles; PGA, 14 micromoles; and dialyzed enzyme, 5.7 mg. in a final volume of 0.8 ml. Incubations were at 37° C.

† The figures in parentheses represent micromoles.

solvent C, and sections of this paper were eluted and counted. During the incubation radioactivity was liberated which, on chromatography, migrated in the area corresponding to inorganic phosphate. The amount recovered was the equivalent of 0.22 micromole of AMP^{32} split in one hour.

DISCUSSION

The experimental results indicate that in this extract of heart muscle and under the conditions employed, the synthesis of purine nucleotides via the *de novo* pathway (reactions 1 to 3, *etc.*) was less efficient than synthesis via utilization of the free base (reaction 4) or via phosphorylation of adenosine (reaction 5). The incorporation of glycine-1-C^{14} into glycinamide ribotide requires at least three enzymatic steps when ribose-5-phosphate, ATP, glutamine and glycine are supplied. At least two steps are required when PRPP is utilized and probably one when PRA is employed (reactions 1 to 3). The stimulation of glycine-1-C^{14} incorporation in the presence of these substrates suggests that the incorporation noted represents purine precursor synthesis in spite of the fact that neither glycinamide ribotide nor formylglycinamide ribotide were isolated. If the glycine incorporation does represent *de novo* synthesis, then 0.006 micromole of purine precursors was synthesized in one hour under these conditions when ribose-5-phosphate and glutamine were used.

Spilman (21) has provided isotopic evidence that the acid soluble adenine nucleotides of dog heart muscle are in a dynamic state. Ninety minutes after injection with C^{14}-formate the dog was sacrificed and the acid soluble nucleotides were isolated and counted (counts per minute per 5 mg. of purine). Activities were: gastrointestinal tract, 680; heart, 467; kidney, 289; liver, 34; while the urine uric acid was 3,150. The remarkably high activity of the heart could represent exchange of the 2 carbon, *de novo* synthesis, or possibly synthesis from hypoxanthine made in the liver and transported through the blood. From the experiment it is impossible to conclude which of these mechanisms may be operative, but the high activity does indicate a dynamic state.

The condensation of a free purine base, either adenine or hypoxanthine, with PRPP to form the corresponding nucleotide (reaction 4) occurs readily in cardiac muscle extracts. In experiment 3, Table II, 0.164 micromole of nucleotide was synthesized in one hour. The synthesis of PRPP may be limiting as only 0.047 micromole of nucleotide was synthesized when ribose-5-phosphate and ATP rather than PRPP were employed. This activity, however, is almost ten times the rate of glycine incorporation under somewhat comparable conditions. This enzymatic mechanism which has been shown to be present in skeletal muscle (22) would require either adenine or hypoxanthine. The demonstration of both of these purine bases in the urine of normal humans (23) suggests that they probably are available via the blood stream to cells of the body. Indeed, hypoxanthine has been identified in plasma (24). That these free bases are utilized for nucleic acid synthesis by tissues such as liver and intestine has been demonstrated by *in vivo* experiments (25, 26). It should be noted that hypoxanthine is not utilized by extracts of bone marrow for the synthesis of purine nucleotides (27). No experiments on purine utilization by cardiac muscle are reported and similar experiments on skeletal muscle are conflicting. The observation in the rat that the amino group of muscle adenylic acid was easily labeled with $N^{15}H_3$ while the ring nitrogens were not (28) suggested that only the amino group was in a dynamic equilibrium. However, when a high level of uniformly labeled N^{15}-adenine was fed, muscle ATP became labeled (24). Comparable levels of labeled hypoxanthine did not result in labeling of the ATP (29). These results suggest that enzymes are present in skeletal muscle for the incorporation of adenine, but that the turnover rate in this tissue is slow.

The conversion of the nucleoside adenosine to a nucleotide, in the presence of ATP and the muscle extract (reaction 5), is indicated in Table V. Assuming the rate of synthesis in the first 15 minutes was maintained for one hour, 0.37 micromole of adenylic acid would be synthesized. This appears to be the most efficient *in vitro* mechanism. The adenosine needed for this reaction might arise from adenine via reaction 8. However, the presence of a strong adenosine deaminase, as indicated by the recovery of inosine and hypoxanthine in experiments 2 and 3, Table V, makes the sig-

nificance of the adenosine kinase difficult to interpret, but differential cellular localization of these enzymes cannot be ruled out. It is of interest that Lowy, Davoll, and Brown (30) have found considerable incorporation of adenosine-8-C^{14} and inosine-8-C^{14} into the ATP of skeletal muscle. Thus the more effective pathways for synthesis of nucleotides in heart muscle extracts appear to be the utilization of the free purine base and of adenosine. Which of the three pathways is utilized predominantly *in vivo* remains to be determined.

With a dynamic state of the nucleotides in heart muscle, one would also expect to find degradative mechanisms operative. An enzyme responsible for deamination of adenylic acid to form ammonia and inosinic acid (reaction 6) was originally described in skeletal muscle (10), but has also been isolated from heart muscle (31). It is most reasonable to suspect that this enzyme was responsible for the recovery of inosinic acid (Table IV). The dephosphorylation of adenylic acid has also been described in heart muscle (31), and evidence is presented in this paper which supports this finding. The enzyme nucleoside phosphorylase (reaction 8) is most likely responsible for the appearance of hypoxanthine (Table V, experiments 2 and 3). It is clear that a potent adenosine deaminase (reaction 9) (Table V, experiments 2 and 3) is present in the pig heart extracts. In view of the ability of adenosine to produce heart block (32) and vasodilatation (33), this enzyme may serve a useful role in protecting and possibly regulating myocardial metabolism.

Thus, enzymatic mechanisms are present for the synthesis and breakdown of nucleotides in heart muscle. The loss of nucleotides from an *in vitro* heart muscle preparation occurs especially under anaerobic conditions and when there is excess potassium (34). In intact perfused hearts, the appearance of inosine and hypoxanthine in the perfusates associated with decreased O_2 tension (35) indicates that under conditions which may pertain to physiopathological states, there may be a loss of nucleotides from the heart muscle. Thus the concept of a dynamic equilibrium of synthesis and degradation of nucleotides in heart muscle and its possible relationship to cardiac function still remain to be explored.

SUMMARY

1. Mechanisms of synthesis and degradation of purine nucleotides in an extract of pig heart muscle have been explored.

2. In this extract the more active synthetic pathways are via the phosphorylation of adenosine and the condensation of adenine or hypoxanthine with PRPP. *De novo* synthesis appears less active.

3. Evidence for the dephosphorylation of adenylic acid as well as for the enzymes adenylic acid deaminase, adenosine deaminase, and nucleoside phosphorylase is presented.

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BIOCHEMICAL CHANGES IN LIVER ASSOCIATED WITH KWASHIORKOR [1]

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Kwashiorkor, the most prevalent form of severe protein malnutrition, is a serious disease, often fatal to young children and especially common from weaning to five years of age. There are insufficient data concerning the chemical changes which take place during development of the disease, although clinical information is extensive. Present knowledge about various aspects of the disease has been reviewed by Trowell, Davies, and Dean (1), by Brock (2, 3) and more recently by Scrimshaw and co-workers (4). Its biochemical characteristics include low levels of serum protein, amylase, alkaline phosphatase, pseudo-cholinesterase, cholesterol, riboflavin and vitamin A. Concomitantly the livers are usually found to be high in fat and low in protein.

Waterlow (5, 6) did pioneering work on enzymes in liver biopsy specimens from malnourished children and reported that hepatic lactic dehydrogenase and cytochrome oxidase of four children in Gambia remained virtually unchanged after treatment whereas pseudo-cholinesterase was initially low but more than doubled on treatment. Later, Waterlow and Patrick (7, 8) studied levels of eight enzymes in liver biopsy samples from a large number of malnourished children in Jamaica on admission to the hospital and after treatment. For cytochrome oxidase, lactic, malic and glutamic dehydrogenases, succinoxidase, DPNH-cytochrome C reductase and transaminase they report unchanged activity following treatment. The only enzyme found to be reduced in activity in the disease was non-specific cholinesterase, which increased on treatment.

Extensive dietary studies in various parts of the world where children suffer from kwashiorkor reveal inadequate intake of protein as well as low dietary levels of other essential nutrients (1, 3). The protein is often of poor quality and therefore unfavorable for the synthesis of tissue protein. If dietary situations exist such that protein and other nutrients necessary for the synthesis of hepatic enzymes are inadequate, the levels of some enzymes in the liver should reflect the lack.

Certain hepatic flavin enzymes, particularly xanthine oxidase (9, 10), D-amino acid oxidase (11, 12), and glycolic acid oxidase (12), are greatly decreased either by riboflavin, protein or caloric deficiency in rats. Xanthine oxidase is also lowered by the lack of a single essential amino acid in the diet (13, 14). The levels of these flavoproteins and of riboflavin might conceivably be related to some of the changes in metabolism which occur in the livers of children with diseases of malnutrition, particularly where dietary protein and riboflavin are low.

This report gives results of biochemical measurements on liver biopsy samples from Guatemalan children with kwashiorkor. Analyses were made for xanthine, D-amino acid, and glycolic acid oxidases, DPNH-dehydrogenase, malic dehydrogenase, transaminase, riboflavin, total oxidized pyridine nucleotides, cholesterol, lipid and protein in the liver as well as the protein, cholinesterase and amylase in serum and riboflavin in red blood

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cells. Samples were obtained from 13 children, in six cases both before and after treatment. For comparison, levels in liver specimens at autopsy from North American children dying of causes unrelated to kwashiorkor were also measured. To assess possible postmortem effects the livers of normal rats were assayed at various intervals after death.

EXPERIMENTAL METHODS

Sampling procedures. The children were selected as cases of typical kwashiorkor on admission to the General Hospital or the Hospital of the "Sociedad Protectora del Niño" of Guatemala City. If the prothrombin time of a blood sample proved satisfactory the initial biopsy sample was taken before any feeding or treatment was given. Franseen needle biopsies were done by aspiration through the pleural space after local anesthesia with procaine. The sample was put at once into an ice-cold tube which remained in ice until brought to the laboratory 30 to 45 minutes later.

The biopsy sample was rapidly weighed and dropped into a small glass homogenizer, containing 15 to 20 volumes of 0.02 M nicotinamide at 4° C., and thoroughly homogenized. Nicotinamide was used to prevent splitting of DPN by tissue DPNase. If the sample was large enough, about 5 mg. were taken for histological study. Each sample weighed 20 mg. on the average and provided enough tissue for the 12 analyses in duplicate or triplicate. Initial biopsy samples were obtained from 10 children. From six of these, biopsy samples were also obtained after three to four weeks of treatment. At this time the children were gaining weight and typical clinical signs of the disease such as edema, diarrhea, and skin lesions had disappeared. Although accurate dietary control was not possible to achieve in the wards of the General Hospital, the children usually received a mixed diet which included milk and supplementary vitamins. Adequate supervision of the diet was possible at the Hospital of the "Sociedad Protectora del Niño" where two children, M.A. and S.R., stayed. They received a diet rich in protein, particularly milk protein, eating gradually increasing amounts up to 5 grams of protein and 150 calories per kilo per day.

No liver samples from well-nourished Guatemalan children with no history of kwashiorkor were available for determinations of normal liver levels of chemical substances. However, to add to the group of recovered cases, biopsy samples were analyzed from three children clinically recovered from kwashiorkor, S.R., T.A. and I.T., from whom no initial samples were obtained. Another approximation of normal hepatic levels was obtained from liver at autopsy of children dying of causes unrelated to kwashiorkor in the St. Louis Children's Hospital. To determine possible consequences of postmortem delay, livers from a series of rats were analyzed at intervals of 0, 1, 6 and 10 hours after death from a blow on the head. The dead rats were allowed to remain at room temperature during the specified time intervals.

Analytical procedures. Labile enzymes were measured as soon as possible after sampling. DPNH-dehydrogenase was determined within 30 minutes after samples arrived in the laboratory. A spectrophotometric method was used which involved measurement of the change in optical density of DPNH at 350 mμ with potassium ferricyanide as the electron acceptor (12). D-amino acid and glycolic acid oxidases were measured by the method of Burch, Lowry, Combs, and Padilla (15) scaled to the use of 0.25 mg. of tissue (30 μl. incubation volumes). These methods depend upon the spectrophotometric measurement of the 3-hydrazinoquinoline derivatives of the α-keto acids formed during the action of the enzymes on D-alanine or glycolic acid, respectively, during a 30-minute incubation period. Xanthine oxidase was measured by the rate of oxidation of 2-amino-4-hydroxypteridine to fluorescent isoxanthopterin in the presence of 5×10^{-3} M methylene blue (12). This method completely avoids the usual troubles from tissue blanks. As a rule, 10 μl. of 1:20 homogenate, or 0.5 mg. of liver were used for each analysis. Riboflavin coenzymes, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) were determined on the first few biopsy samples and the autopsy samples by a published fluorometric procedure (16). On later biopsy samples only the total riboflavin was measured. Oxidized pyridine nucleotides (PN) were determined after trichloroacetic acid precipitation of protein by a fluorometric procedure of Lowry, Roberts, and Kapphahn (17). Protein was determined colorimetrically (18).

The more stable enzymes and other substances were measured on frozen aliquots of the homogenates. For malic dehydrogenase (MDH) and aspartic-glutamic transaminase, methods developed for brain enzymes (19, 20) were adapted to liver samples. MDH was allowed to reduce oxalacetate with DPNH, and the DPN+ formed was measured by its fluorescence in alkaline solution. Transaminase was allowed to act on α-keto glutarate and aspartate in the presence of DPNH and an excess of purified pig heart MDH. The oxalacetate produced immediately oxidized DPNH to DPN+ which was measured fluorometrically. For each determination of MDH 0.15 γ of human liver or 0.08 γ of rat liver were incubated in a 12-μl. volume, and for each determination of transaminase 5 γ of human or rat liver were incubated in 50 μl.

Cholesterol in 0.5-mg. liver samples was estimated by the fluorometric method of Albers and Lowry (21) modified by McDougal and Farmer (22) for serum. Good reproducibility and 98 to 100 per cent recovery of added cholesterol were obtained. Lipids were measured on samples of similar size. Extraction was accomplished with 3:1 alcohol-ether mixture by adding 10 volumes for each of three extractions. The solvents were evaporated in a water bath at 90° C. and finally the last traces were removed in a special vacuum desiccator (21). The residue was extracted with ether; the ether was evaporated

TABLE I

Hepatic riboflavin and flavin enzymes in kwashiorkor, initially (I) and after treatment (T) *

Case	Sex and age yrs.	Clinical severity	Time treated days	Total riboflavin mg./Kg.		Xanthine oxidase mM/Kg./hr.		D-amino acid oxidase mM/Kg./hr.		Glycolic acid oxidase mM/Kg./hr.		DPNH-dehydrogenase M/Kg./hr.	
				I	T	I	T	I	T	I	T	I	T
P. G.†	F-2.0	sev.	4	26		0.0		55		71		4.2	
R. O.†	M-5.5	mod.	10	137		1.7		134		473		17.5	
M. J.	F-3.0	mod.	12	138		1.6		78				20.1	
L. A.	F-7.0	mod.	17	108		3.5		147		362		16.3	
Z. A.	F-3.8	mod.	26	123	109	1.5	7.9	114	581	485	527	20.2	15.5
M. A.	M-4.0	mod.	48	155	109	4.0	5.2	168	552	500	524	24.2	15.4
R. V.	F-1.8	mod.	28	142	99	3.1	7.6	218	444	764	456	12.7	15.5
M. C.	F-1.7	mod.	29	92	122	2.7	5.4	179	430	342	509	15.0	14.4
L. S.	F-4.0	sev.	29	113	106	3.9	6.4	216	218	506	221	19.6	12.6
L. C.	F-3.0	sev.	26	103	115	1.2	5.2	96	421	333	516	21.0	17.2
S. R.	M-1.5	mod.	60		138		10.5		433		639		17.6
T. A.	M-1.5	mod.	168		113		8.0		407		575		14.5
I. T.	M-3.3	sev.	71		103		5.6		402		587		15.6
	Mean			123	113	2.6	6.9	150	432	471	506	18.5	15.4
	S.E.			7	4	0.4	0.6	16	34	52	42	1.2	0.5

* Values expressed per Kg. of liver protein.

† These two children died in the hospital. Values for P. G. were much lower than all others and have been excluded from the averages.

off and the lipid determined by the colorimetric method of Bragdon (23) adapted for 8 to 100 μg. of lipid in a final volume of 0.4 ml. by Chiang, Gessert, and Lowry (24). Trimyristin (Distillation Products Industries) was used as the standard lipid. Quantitative recovery of added lipid and reproducibility were obtained in preliminary tests made with rat and human liver.

In serum, protein was measured by the gradient tube method of Lowry and Hunter (25), cholinesterase and amylase by methods of Reinhold, Tourigny, and Yonan (26) and Smith and Roe (27). Red blood cell riboflavin on packed red cells was measured by a previously published method (28).

RESULTS

A. Clinical findings

Four of the children had severe kwashiorkor and the remainder were cases of moderate severity. All showed typical hair changes, skin lesions and edema. Apathy was a general characteristic, and all had diarrhea. Child P.G. was diagnosed initially as having very severe and apparently irreversible kwashiorkor and died four days after entering the hospital.

B. Hepatic flavin enzymes

All enzyme and coenzyme values have been calculated on the basis of protein. The data on riboflavin and four flavin enzymes (Table I) obtained on six children from whom biopsy samples were taken initially and after treatment revealed no significant rise in riboflavin, glycolic acid oxidase or DPNH-dehydrogenase. Remarkable increases are apparent in xanthine oxidase and D-amino acid oxidases. These findings are of special interest since, in the rat, hepatic xanthine oxidase falls with low dietary levels either of protein, calories or riboflavin, whereas DPNH-deyhdrogenase does not change in caloric restriction or riboflavin deficiency (12) until animals are near death. D-amino acid and glycolic acid oxidases in rat liver also fall if dietary riboflavin is absent but are not so greatly affected by low protein or calories as xanthine oxidase. Therefore, D-amino acid oxidase may not respond in the same manner to dietary restrictions in children with kwashiorkor as it does in rats.

Unusually low levels were obtained on child P.G. who failed to respond to treatment. Possibly the very low levels of these enzymes and other substances were associated with irreversible changes in the liver. The liver sample of P.G. was more fatty in gross appearance than any other. The DPNH-dehydrogenase is particularly interesting as it is the only really low value found for this enzyme.

The values in Table I indicate a highly significant increase in hepatic xanthine and D-amino acid oxidase levels calculated on the basis of liver protein of eight children before and after treatment of kwashiorkor. The other enzymes given in Tables I and II did not increase significantly relative to liver protein.

C. Hepatic substances other than flavin enzymes

Liver protein (Table II) increased on an average of 38 per cent ($p < 0.01$) during treatment in the six individuals studied both before and after therapy. At the same time total lipid fell to less than one-third the initial value, whereas cholesterol in the few samples tested showed no significant change. Two non-flavin enzymes, transaminase, and malic dehydrogenase, chosen for their significance in relation to amino acid metabolism and the citric acid cycle, respectively, were measured as controls of the flavin enzymes. Neither enzyme was changed significantly in the liver after treatment.

Pyridine nucleotides (DPN and TPN) appear to increase in most cases upon treatment although the increase is not statistically significant. These nucleotides are destroyed with exceptional speed by tissue and red cell enzymes and may have been partially split in some samples before analysis was possible. A change in extent of oxidation of these coenzymes would also affect the results. In rat liver samples, a decrease in total oxidized pyridine nucleotides PN occurred when they were kept in an ice bath at 4° C. for one hour without nicotinamide and analyzed by the technique used on the biopsy samples (Table IV). Therefore, the levels reported here are possibly somewhat low but should be comparable before and after treatment since the specimens were similarly handled, and enzyme action was stopped by adding aliquots to trichloroacetic acid at once.

D. Protein and enzymes in serum

A significant increase ($p < 0.01$) in serum protein on treatment (Table III) was accompanied by a significant rise ($p < 0.01$) in serum cholinesterase relative to protein. The increase in serum amylase was not significant. If activities of these enzymes are calculated per unit volume the increase become three-fold for cholinesterase and two-and-one-half-fold for amylase. Thus they would appear to have decreased similarly in kwashiorkor and to be synthesized at approximately the same rate upon recovery from the disease. It is obvious from the data, however, that the two enzymes behave differently relative to serum protein.

TABLE II

Liver concentrations of certain substances in kwashiorkor, initially (I) and after treatment (T) *

Case	Protein Gm./Kg.* I	Protein Gm./Kg.* T	Lipid Gm./Kg.† I	Lipid Gm./Kg.† T	Cholesterol Gm./Kg.† I	Cholesterol Gm./Kg.† T	Malic. dehyd. M/Kg.†/hr. I	Malic. dehyd. M/Kg.†/hr. T	Transaminase M/Kg.†/hr. I	Transaminase M/Kg.†/hr. T	Oxidized PN mM/Kg.† I	Oxidized PN mM/Kg.† T
P. G.†	31						19		29		0.7	
R. O.	108		995				89		85		3.8	
M. J.	167						102		91			
L. A.	141						107		64		2.8	
Z. A.	124	150		257		23	101	132	96	76	2.8	5.9
M. A.	106	199	805		24		121	90	91	50	4.5	3.4
R. V.	87	192	833	250	28	25	176	113	91	60	3.4	4.1
M. C.	133	169	737	190	30	15	108	81	64	60	3.0	3.6
L. S.	121	167	731	219	15	24	122	105	84	56	4.1	4.6
L. C.	147	186					115	106	69	83	3.0	4.3
S. R.		144		360		26		124		118		5.3
T. A.		172		194		16		102		66		4.1
I. T.		191		199		18		100		70		3.8
Mean	126	174	820	238	24	21	116	106	82	71	3.4	4.4
S.E.	8	6	47	19	2	2	8	6	4	7	0.3	0.3

* Protein per Kg. of wet tissue; others expressed per Kg. of liver protein.
† Values for child P. G. were much lower than all others and have been excluded from the averages.

TABLE III

Levels of four constituents of blood serum or cells in kwashiorkor and the effects of treatment

Case	Serum protein *Gm./100 ml.*		Serum cholinesterase *units/Gm. protein**		Serum amylase *Smith-Roe units/Gm. protein*		Red cell riboflavin *μg./100 ml.*	
	I	T	I	T	I	T	I	T
P. G.	3.45†		275†		1.7†		24.2†	
R. O.	3.77		250		13.3		10.5	
L. A.	4.60		335		3.0		14.7	
Z. A.	4.10	6.68	330	540	8.3	11.1	11.2	18.6
M. A.	4.36	6.91	235	890	85.0‡	7.5‡	10.9	19.2
R. V.	4.50	7.68	455	935	2.9	15.0	11.0	
M. C.	3.50	6.00	270	550	3.1	8.0	23.8	27.8
L. S.	3.90	6.42	400	610	20.0	12.4	24.0	39.0
L. C.	3.74	7.48	515	660	8.3	11.4	13.9	24.2
S. R.								23.2
T. A.		6.40		820		4.5		
I. T.		6.75		715		14.8		26.2
Mean	4.06	6.79	350§	715§	8.4§	11.0§	15.0	25.4
S.E.	*0.14*	*0.20*	*30*	*55*	*2.3*	*1.5*	*2.0*	*2.3*

* The unit activity of the original serum as defined by Reinhold, Tourigny, and Yonan (26) multiplied by 5,000 and divided by Gm. protein per 100 ml.

† Omitted from the average as in previous tables.

‡ Child M. A. had hypertrophied salivary glands. These values have not been included in the average.

§ The average serum cholinesterase expressed as Michel Units is 0.28 ± 0.03 initially and 0.98 ± 0.09 after treatment; the serum amylase expressed as Smith-Roe Units per 100 ml. is 33 ± 9 and 76 ± 11, respectively.

Comparison of the increases in serum protein and cholinesterase with that of liver protein (Table II) suggests that serum cholinesterase and serum protein regenerate simultaneously with liver protein during recovery. The reports of Waterlow and others (1) that cholinesterase and protein in both liver and plasma are low in kwashiorkor and increase greatly on treatment are in accord with these results. The data on serum amylase are too variable to indicate any significant relation to protein in liver.

Red blood cell riboflavin increased 70 per cent on treatment although the liver riboflavin failed to show such a change. Few data are available for red cell riboflavin levels in young children, but for four children of similar ages an average of 30 γ per 100 ml. was previously reported (29). In adults, Bessey, Horwitt, and Love (30) have found the red cell level to be a reliable criterion of riboflavin deficiency, although in rats, at least, the liver is a more sensitive index of mild deficiency (31). Since there was no other evidence of riboflavin deficiency, the changes may be secondary to other alterations in the red cells.

E. Liver specimens at autopsy

Since information similar to that obtained from livers of children with kwashiorkor was not available for those of normal young children, measurements were made on liver specimens from St. Louis autopsies. Samples from five children who seemed to be well-nourished and who died after periods of one to four days in the hospital were analyzed. The time before samples were available varied from two to six hours after death. Levels obtained on the liver autopsy samples (Table IV) are in most instances similar to those obtained on liver biopsy samples of the cases of kwashiorkor after treatment. However, xanthine oxidase and glycolic acid oxidase activities averaged distinctly higher in the autopsy samples (100 and 50 per cent, respectively). The average xanthine oxidase value is five times greater than for the untreated children with kwashiorkor. Since glycolic acid oxidase in kwashiorkor did not change appreciably as the result of treatment, the low values among the children concerned may reflect some deficiency or disease state other than kwashiorkor. For example, this enzyme is a sensitive index of riboflavin deficiency (12).

TABLE IV

Enzyme activities of human and rat liver at autopsy *

Child† and age	Time to autopsy	Protein	FAD	FMN‡	Total riboflavin	Xanthine oxidase	D-amino acid oxidase	Glycolic acid oxidase	DPNH-dehyd.	Malic dehyd.	Trans-aminase	Oxidized PN
		Human liver										
	yr.	*hr.*	*Gm./Kg.w*	*mg./Kg.p*	*mg./Kg.p*	*mg./Kg.p*	*mM/Kg.p/hr.*	*mM/Kg.p/hr.*	*mM/Kg.p/hr.*	*M/Kg.p/hr.*	*M/Kg.p/hr.*	*M/Kg.p/hr.*	*mM/Kg.p/hr.*
R. J. B.,	3.0	6	155	99	7.4	106	8	271	562	10.8	121	53	1.8
E. H. L.,	2.0	3	120	91	11.1	102	9	396	892	15.2	115	53	4.8
W. G. H.,	7.0	2	175	90	13.7	104	14	351	732	17.8		65	3.1
R. J.,	2.5	3	162	68	4.3	72	18	505	617		281	110	3.7
D. A. Y.,	9.0	4	150			115	17	339	867	19.0	115	61	4.1
	Mean		152	87	9.1	100	13	372	734	15.7	158	68	3.5
	S.E.		*11*	*8*	*1.8*	*7*	*2*	*37*	*65*	*2*	*36*	*10*	*0.5*
		Rat liver										
No. of rats												
3		0	191§	150	14.0	164	48	814	920	35.0	200‖	86	4.9
			4	*5*	*1.4*	*6*	*2*	*27*	*94*	*2.3*		*2*	*0.5*
3		1¶	186	148	12.2	161	50	785	918	38.2			3.6
			4	*3*	*1.7*	*4*	*2*	*33*	*75*	*0.4*			*0.3*
3		1	187	132	19.0	151	43	874	720	38.9		76	4.1
			8	*8*	*2.0*	*10*	*2*	*76*	*135*	*1.2*		*5*	*0.4*
3		6	181	132	22.0	153	46	694	758	32.6			3.3
			5	*3*	*0.3*	*3*	*4*	*84*	*62*	*0.1*			*0.3*
3		10	181	128	26.0	154	43	730	877	29.7			2.7
			1	*3*	*2.3*	*7*	*2*	*42*	*74*	*0.6*			*0.4*

* Values are expressed as in Tables I and II. FMN and FAD are calculated as mg. riboflavin per kilo of protein.

† The causes of death were: acute bacterial meningitis, R. J. B.; pneumonia and cerebral edema of undetermined etiology with hypoxic nephrosis and moderate fatty metamorphosis of the liver, E. H. L.; tumor of the left lateral ventricle, W. G. H.; congenital heart disease with renal tubular acidosis and chronic congestion of the liver, R. J.; cerebral tumor, D. A. Y. Except for R. J., these cases had an acute onset of symptoms and died within a few days.

‡ The small amount of free riboflavin in liver is included in these values.

§ Figures are means with the standard error given below.

‖ This is the average of three adult rats.

¶ These analyses were done on liver samples from the first rats (zero time) after storage in a tube at 4° C. for one hour.

The rat liver analyses (Table IV) are presented as a confirmation of the validity of analyses on autopsy material. None of the four enzymes tested were diminished significantly within six hours of the death of the rat, and only one, DPNH-dehydrogenase, was changed within 10 hours. The coenzymes are not so stable; 10 to 15 per cent FAD was converted to FMN in 6 to 10 hours, and pyridine nucleotides were either hydrolyzed or reduced by 40 per cent in 10 hours. Human liver may not split FAD as rapidly as rat liver, however, since FMN was only 10 per cent of FAD in spite of the time lag before obtaining specimens. It will be noted that total riboflavin and all four flavin enzymes are higher in rat liver than in human liver, although transaminase is only slightly higher in the rat.

DISCUSSION

Values for liver and serum enzyme activities have been expressed throughout on the basis of protein because of the well known changes in liver and serum protein concentrations during treatment of kwashiorkor.

Hepatic flavin enzymes

The flavin enzymes, xanthine and D-amino acid oxidase, which were found to be low in kwashiorkor have also been shown to be lowered in rat liver by either protein or riboflavin deficiency. Although glycolic acid oxidase is greatly diminished in rat liver by riboflavin deficiency, it remains unchanged in human liver with treatment of kwashiorkor.

DPNH-dehydrogenase, however, is not altered in either the liver of children with kwashiorkor or in the tissues of rats during riboflavin deficiency or caloric restriction. Wainio and co-workers (32) report that DPNH-cytochrome C reductase falls in protein deficiency in the rat, but this has not been confirmed in this laboratory (12). Its failure to fall in these livers, except in the child P.G. who died, supports the idea that this is one of the most crucial of known flavin enzymes and one of those most firmly held by liver tissue even in various deficiency states.

This seeming paradox of finding no fall in riboflavin, relative to protein in liver from kwashiorkor cases when some flavin enzymes have dropped, is resolved by the fact that these enzymes account for an exceedingly small fraction of the total flavin present in liver.

Other substances in the liver

It is of interest that cholesterol does not increase in the liver in kwashiorkor according to the values presented here. This is contrary to the few data available on cases of kwashiorkor from India (33) in which much higher initial levels were found and a decrease with treatment was noted.

The finding of virtually unchanged levels of hepatic malic dehydrogenase and transaminase activity upon treatment of kwashiorkor agrees with Waterlow and Patrick (7). However, the absolute activity found by these investigators for transaminase is only one-fifteenth of the level reported here. This discrepancy may be traced to differences in methods used. These workers also found rat liver to be seven times lower in transaminase activity than human liver. In the method used here, oxalacetate produced is immediately reduced to malate, which prevents approach to equilibrium and consequent slowing of the reaction. Without means for removing one of the products, larger samples as may have been used by Waterlow and Patrick (7) in their studies of rat liver would give lower relative values.

It is now possible to measure a large number of enzymes with relative ease in the amount of material obtained by liver needle biopsy specimens. Through study of alterations in the enzymes, a better understanding of the metabolic changes in diseased tissues may be achieved. Changes in these functional proteins are particularly relevant to a deficiency disease such as kwashiorkor.

SUMMARY

1. Chemical changes in liver and serum during treatment of 13 children with kwashiorkor are reported. Results of similar analyses on liver autopsy specimens from five St. Louis children dying from causes unrelated to kwashiorkor are also included, together with analyses of livers from 12 rats, to confirm the validity of studying autopsy material.

2. The increase in protein and the decrease in total lipid with treatment were marked. Concomit-

antly there were striking increases relative to protein in xanthine oxidase and D-amino acid oxidase.

3. During treatment of the children with kwashiorkor no significant changes *relative to protein* were found in the following substances in liver: riboflavin, glycolic acid oxidase, DPNH-dehydrogenase, malic dehydrogenase, transaminase, oxidized pyridine nucleotides, and cholesterol.

4. The levels of all substances measured in the livers of kwashiorkor cases after treatment were generally equal to those of autopsy specimens of St. Louis children, except for glycolic acid oxidase and xanthine oxidase which did not increase to the values found in the autopsy samples.

5. Protein in serum increased 70 per cent during treatment and relative to protein, cholinesterase increased 100 per cent and amylase 30 per cent. The red blood cell riboflavin doubled.

ACKNOWLEDGMENTS

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FURTHER CHARACTERIZATION OF AN INSULIN ANTAGONIST IN THE SERUM OF PATIENTS IN DIABETIC ACIDOSIS

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We have previously described an insulin antagonist in the sera of patients in diabetic acidosis (1, 2). The protein nature of this material was suggested by its lability at 100° C. and its electrophoretic migration with the α-globulins. The antagonist was not a lipoprotein and did not exhibit any insulinase activity. Its inhibitory effect was still demonstrable whether it was added to the hemidiaphragm preparation before or after exposure to insulin. Experiments using insulin-I^{131} indicated that the antagonist did not compete with insulin for binding sites on diaphragm (2).

Further characterization of this insulin antagonist serves as the basis for this report.

METHODS AND MATERIALS

The method for determining the insulin effect was the same as was used previously and depends on the ability of insulin to augment glycogen deposition by the rat hemidiaphragm (1). In the presence of normal serum the insulin effect has been found to be 5.35 micromoles of glycogen (glucose equivalent) per gram of tissue (1).

Serum or plasma used in these experiments was obtained from patients in diabetic acidosis at the time of their admission to the hospital, before or shortly after insulin therapy was started.[1] Samples were kept frozen for over one year without any evident loss of insulin antagonist activity.

Trypsin digestion of insulin antagonist serum was accomplished by the addition of 12 mg. of crystalline trypsin (Worthington Biochemical Corp.) to 6 ml. of serum followed by incubation at room temperature for four hours. Because of the large excess of trypsin present, the hemidiaphragm assay procedure was modified slightly to prevent digestion of the insulin in the system. Each hemidiaphragm was added to 1 ml. of trypsin-digested serum and 1 ml. of 0.04 M phosphate buffer, pH 6.8, and the mixture was agitated for five minutes using a stream of oxygen. The hemidiaphragms were then removed from this mixture and washed twice with buffer. While one hemidiaphragm was exposed to 0.2 unit of insulin in 2 ml. of buffer for one minute, the other was put in buffer without insulin. Incubation was then carried out as usual.

Chymotrypsin digestion was achieved by adding 12 mg. of crystalline chymotrypsin (Worthington Biochemical Corp.) to 6 ml. of insulin antagonist serum and incubating at room temperature for seven hours. At the end of this time 0.008 ml. of pure diisopropyl fluorophosphate (DFP) was added and the incubation continued for another 30 minutes. The addition of DFP was necessary to prevent the inactivation of insulin by chymotrypsin during the subsequent assay.

Serum was separated into its various protein components by starch block electrophoresis according to the method of Kunkel and Slater (3).[2] Potato starch previously washed with barbital buffer (pH 8.6) was used. The electrophoresis of serum was carried out for 18 hours at 4° C., using 350 volts and 150 milliamperes. The starch block was then cut up into segments, each of which was washed with buffer and centrifuged. The clear supernatant solution was then decanted. The various protein components were identified by plotting the protein concentration of each tube against the tube number as shown in Figure 1. The protein was determined according to the modified method of Folin (4). In addition, albumin, α_2-globulins, and β-globulins were located on the starch block by their distinctive color bands —yellow, greenish red, and rust, respectively. The contents of the tubes were then pooled as indicated in Figure 1 to give the five different protein fractions tested. The amounts of α_1-globulins and other protein fractions in sera from patients in diabetic acidosis were measured by paper electrophoresis using the method of Jencks, Jetton, and Durrum (5).[3] The dry paper strip patterns were evaluated by transmission densitometry.[4] Each electro-

[1] We are indebted to Dr. F. Bruce Peck, Jr., Eli Lilly and Company, Dr. James Craig, Western Reserve Medical School, Drs. James Pittman and John Binion, University of Alabama Medical School, Dr. Dorothy Macy, Jr., Philadelphia General Hospital, Drs. Richard Eberley and Kenneth Young, Jefferson Medical School, Dr. William Morgan, Massachusetts General Hospital, and Dr. James Moss, Georgetown University Medical School, for their helpful cooperation in obtaining sera from patients in diabetic acidosis.

[2] We are indebted to Dr. Edward C. Franklin of the Rockefeller Institute for Medical Research for doing the protein fractionation.

[3] Dr. John Fahey of the National Cancer Institute kindly carried out these determinations.

[4] Analytrol, Spinco Division, Beckman Instruments, Inc., Belmont, California.

phoretic run in a given cell included at least one strip which contained 0.008 ml. of a standard normal human serum.

Glucagon activity *in vivo* was measured in fed male rabbits weighing approximately 2 Kg. After a baseline for blood glucose concentration had been obtained, 0.1 ml. of normal serum containing 0.05 mg. of glucagon was injected rapidly into an ear vein. Fifteen minutes later the blood glucose was again determined by the micromethod of Folin (6).

The human insulin employed in this study was prepared essentially according to the method of Romans, Scott, and Fisher (7) with modifications appropriate for the handling of smaller quantities of starting material. Three hundred grams of human pancreas, obtained at autopsy, were extracted first with acidified ethanol and then at pH 8, according to the published procedure (7). After adjustment of the pH to 5.3, the solution was rendered lipid-free by the method of Light and Simpson (8). The resultant precipitate was dissolved in 75 ml. of 0.05 N hydrochloric acid and sodium chloride was added to a concentration of 25 per cent (w/v). The precipitate obtained upon centrifugation was dissolved in 25 ml. of 0.5 M acetic acid and the pH of the solution adjusted to 5.9 with 0.5 M ammonium hydroxide. The suspension was refrigerated overnight and the precipitate was collected by centrifugation and redissolved in 25 ml. of 0.5 M acetic acid. Final precipitation of the hormone was effected as before by adjustment of the pH to 5.9 with 0.5 M ammonium hydroxide. The precipitate was collected by centrifugation, dissolved in 25 ml. of 0.05 N hydrochloric acid and, after overnight dialysis against distilled water, was lyophilized, yielding 21 mg. of salt-free product. Concurrently, the supernatant fluids resulting from centrifugation of the ammonium acetate precipitates were combined and also dialyzed against water and lyophilized. The yield of this product was 72 mg. Examination of the preparations by paper chromatography (8) revealed, surprisingly, that while both products contained detectable amounts of insulin, that which had been recovered from the ammonium acetate supernatant fractions contained a higher proportion of the hormone. The latter preparation was kindly assayed through the cooperation of Dr. Otto K. Behrens of the Lilly Research Laboratories, and found to possess an activity of 1.8 units per milligram. This material was employed in these studies without further purification.

Monkey growth hormone was a generous gift from Dr. C. H. Li, University of California. Glucagon was kindly supplied by Eli Lilly and Company.

RESULTS

Table I indicates that trypsin digestion does not alter the ability of serum from Case IX (2) to inhibit the insulin effect. Normal serum subjected to trypsin digestion did not produce any significant reduction of the insulin effect. Chymotrypsin digestion, however, did inactivate the insulin antagonist in the serum from this same patient (Table II). Since DFP had been added to the serum after the chymotrypsin digestion it was important to determine its effect on the insulin antagonist. The findings indicate that DFP, by itself, does not inactivate the humoral insulin inhibitor.

TABLE I

Failure of trypsin digestion to inactivate serum insulin antagonist

Treatment of serum	Amount of serum	Insulin effect (micromoles [glucose equiv.] per gram of tissue)	No. of determinations
	ml.	Mean ± S.E.M.	
Trypsin digested serum, Case IX	1	−0.03 ± 0.84	3
Untreated serum, Case IX	1	1.40 ± 0.43	8
Trypsin-digested normal serum	1	4.28 ± 0.85	12

In an earlier paper the insulin antagonist was reported to reside in the α-globulin fraction of the serum proteins (2). At that time it could not be determined whether it was localized in the α_1 or the α_2 fraction. However, using starch block electrophoresis it was possible to obtain some separation of these two protein fractions. In confirmation of our previous results, no antagonist activity was demonstrable in the albumin or the γ-globulin fractions. It is apparent from Table III that the bulk of the insulin antagonist activity was in the α_1-globulin fraction of the serum proteins. At a concentration equal to that of original

TABLE II

Inactivation of insulin antagonist by chymotrypsin digestion

Treatment of serum	Amount of serum	Insulin effect (micromoles [glucose equiv.] glycogen per gram of tissue)	No. of determinations
	ml.	Mean ± S.E.M.	
Untreated serum, Case IX	1.00	1.40 ± 0.43	8
Chymotrypsin + DFP + serum, Case IX	1.00	7.79 ± 1.98	3
Untreated serum, Case VII	0.05	1.03 ± 1.32	3
DFP + serum, Case VII	0.05	1.13 ± 1.68	3

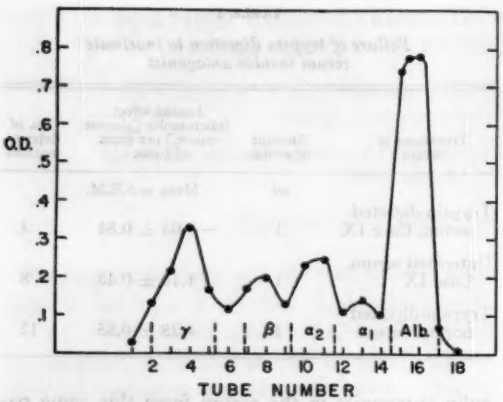

FIG. 1. ELECTROPHORETIC SEPARATION OF COMPONENTS OF ACIDOTIC SERUM

serum there was suggestive activity in the α_2-globulins as compared to albumin, β- and γ-globulins. The slight activity in the α_2-globulin fraction was probably attributable to some contamination by α_1-globulins since the separation of these two protein components obviously was not complete (Figure 1). The serum used for this experiment was the same which had been used for the previous protein fractionation (2). The earlier finding of some antagonist activity in β-globulins was undoubtedly due to less complete separation of serum proteins obtained in that experiment.

Since the insulin antagonist activity was contained in the α_1-globulin fraction of the serum proteins, it seemed pertinent to determine whether the amount of this protein component was increased in sera exhibiting insulin antagonism. Scheurlen (9) has previously reported elevated levels of α_1- and α_2-globulins in the sera of some diabetics during acidosis and infection. Table IV

TABLE III

Activity of various protein fractions of insulin antagonist serum

Fraction	Equivalent amount of whole serum	Insulin effect (micromoles [glucose equiv.] per gram of tissue)	No. of determinations
	ml.	Mean ± S.E.M.	
Albumin	0.15	7.23 ± 0.74	3
α_1-globulins	0.15	1.41 ± 0.99	3
α_2-globulins	0.15	4.72 ± 2.26	3
β-globulins	0.15	8.52 ± 1.77	3
γ-globulins	0.15	8.17 ± 2.64	3

compares the distribution of the serum protein fractions in four cases of diabetic acidosis having insulin antagonist and six other patients in diabetic acidosis without such antagonist as measured by the present assay. Whereas considerable variations in the concentrations of protein fractions were encountered, in none of the four samples obtained from diabetics in ketosis which exhibited detectable antagonist did the values for α_1-globulins fall outside the normal range. The failure to find increased α_1-globulins is not necessarily inconsistent with the observation that the antagonist activity resides in that fraction. The α_1-globulins are a heterogeneous group of proteins of which the insulin antagonist may be a minor component. Even a relatively large increase in the latter material might therefore not be evident from electrophoretic studies.

Since Snedecor, DeMeio, and Pincus (10) reported inhibition of the insulin effect on the rat hemidiaphragm by glucagon, the possibility existed that this substance was responsible for the insulin antagonism observed during diabetic acidosis. Table V indicates that the addition of as much as 0.1 mg. of glucagon to 1 ml. of normal

TABLE IV

Serum electrophoresis patterns in diabetic acidosis

Serum	Antagonist present	Total protein	Albumin	α_1	α_2	β	γ
		Gm. %	*Gm. %*	*Gm. %*	*Gm. %*	*Gm. %*	*Gm. %*
Case I	+	5.0	1.63	0.49	0.90	0.63	1.36
Case III	+	8.1	3.12	0.34	0.90	1.93	2.01
Case VII	+	5.2	2.15	0.34	0.71	0.81	1.18
Case IX	+	7.0	3.11	0.35	1.34	1.27	0.92
Six other cases of diabetic acidosis	−	5.6–6.7	1.34–3.18	0.27–0.54	0.56–1.26	0.61–1.67	0.96–2.35
Normal	−		2.91–4.07	0.24–0.50	0.46–1.10	0.38–1.18	0.68–1.86

serum did not significantly lower the insulin effect. Further evidence that the antagonist is not glucagon is summarized in Table VI. When rabbits were injected intravenously with 0.1 ml. of normal serum containing 0.05 mg. of glucagon there was an average rise of 63 mg. per cent in their blood glucose levels within 15 minutes. An equal amount of normal serum or serum from Case VII did not produce any increase in the blood glucose. It had previously been shown that this amount of serum from Case VII was capable of inhibiting 0.2 unit of insulin (1).

Earlier it had been shown that sera obtained from acromegalic patients with diabetes did not inhibit the effect of 0.2 unit of insulin (2). Even though these patients demonstrated insulin resistance as measured by the intravenous insulin tolerance test using 0.1 unit per Kg. of weight, it was possible that they did not at that time have very high levels of circulating growth hormone. The *in vitro* effect of monkey growth hormone on insulin action in the rat hemidiaphragm is shown in Table VII. This preparation of growth hormone had been shown to be metabolically active in humans (11). When used in a concentration of 160 μg. per ml. of normal serum, this growth hormone preparation did not antagonize insulin. In view of the finding of Vallance-Owen and Lukens (12) that the insulin antagonism observed in the depancreatized cat was dependent on both pituitary and adrenal hormones, the *in vitro* effects of both monkey growth hormone and hydrocortisone were evaluated in the hemidiaphragm test system. This combination did not exert any inhibition of the insulin effect (Table VII). Serum was obtained from a patient with hypopituitarism who had been given human growth hormone for the preceding three days. As a result of this administration, the patient developed some evidence of insulin resistance as measured by the glucose-insulin tolerance tests (13). Nonetheless, serum obtained on the last day of growth hormone administration did not exhibit any insulin antagonism (Table VII). Although these observations make it unlikely that growth hormone is responsible for the insulin resistance during diabetic acidosis, this possibility is not completely ruled out. Growth hormone as obtained from the pituitary gland and used *in vitro* may not be the native hormone or produce all the metabolic effects usually ascribed to that hormone.

All the previously described experiments were performed using a commercially available mixture of beef and pork insulins. Although insulin antagonist activity had been found in the sera of patients in diabetic acidosis who had never had prior insulin therapy (1), it still seemed pertinent to ascertain the effect of this antagonist using human insulin in the assay system. Table VIII indicates that the insulin antagonist is just as effective against human insulin as against beef-pork insulin. It is thus conceivable that this insulin antagonist could be of importance in the etiology of some

TABLE V

Inability of glucagon to inhibit the insulin effect

Source of serum	Amount of serum	Insulin effect (micromoles [glucose equiv.] per gram of tissue)	No. of determinations
	ml.	Mean ± S.E.M.	
Normal serum	1	5.35 ± 0.48	23
Normal serum + 0.1 mg. glucagon	1	4.42 ± 0.93	9

TABLE VI

Absence of glucagon effect of insulin antagonist serum

Serum injected into rabbit	Amount	Blood sugar change in 15 minutes
	ml.	*mg. %*
Normal serum and 0.05 mg. glucagon	0.1	63
Normal serum	0.1	−14
Insulin antagonist serum, Case VII	0.1	− 1

TABLE VII

Failure of growth hormone to inhibit insulin effect

Material tested	Amount	Insulin effect (micromoles [glucose equiv.] per gram of tissue)	No. of determinations
	ml.	Mean ± S.E.M.	
Monkey growth hormone, 160 γ/ml.	1	6.49 ± 1.60	3
Monkey growth hormone, 160 γ/ml. + Hydrocortisone, 4 γ/ml.	1	7.70 ± 2.10	3
Serum from patient given human growth hormone	1	8.37 ± 1.41	3

TABLE VIII

Inhibition of human insulin by insulin antagonist during diabetic acidosis

Serum	Type of insulin	Insulin effect (micromoles [glucose equiv.] glycogen per gram of tissue)	No. of determinations
		Mean ± S.E.M.	
Diabetic acidosis	Beef-pork, 0.2 unit/ml.	1.32 ± 2.02	3
Diabetic acidosis	Human, 0.2 unit/ml.	−0.30 ± 1.58	3
Normal	Human, 0.2 unit/ml.	6.48 ± 0.06	3

cases of human diabetes. This consideration derives support from the observation of Vallance-Owen, Hurlock, and Please (14) that insulin added to plasma from diabetic patients requiring insulin and having a high blood sugar, but not in ketosis, did not increase the glucose utilization of the rat diaphragm. However, it is possible that during the process of preparation, the human insulin undergoes some modification which makes it less species specific than endogenous human insulin. Immunologically it has been shown that there is cross-reaction between antibodies prepared using beef insulin and human insulin (15).

DISCUSSION

It is still not possible to identify the exact nature or source of the insulin antagonist which can be detected in the sera of some patients in diabetic acidosis. The protein nature of the antagonist is indicated by its heat lability, electrophoretic migration, and the fact that it is non-dialyzable. Failure of trypsin digestion to inactivate the antagonist suggests that either arginine or lysine is not present in the molecule or is not in a position important to antagonist activity. Due to its broader specificity, chymotrypsin probably produces greater digestion of the substance with consequent loss of activity. It is still possible, though unlikely, that the active substance is a smaller molecule tightly bound to a protein and dependent on this combination for its activity.

Growth hormone, adrenal cortical steroids, and glucagon did not exhibit insulin antagonism in the rat hemidiaphragm system. Although these experiments tend to eliminate these substances as the cause of insulin antagonism, it is still possible that one or more of them, singly or in combination, may be related to the insulin antagonist in some way. This is suggested by the observations of Vallance-Owen and Lukens (12) that depancreatized cats have a humoral insulin antagonist. The antagonist described by these workers was not present if the animal was adrenalectomized or hypophysectomized. The inhibitor could be restored by administering cortisone or hydrocortisone for four days. However, since a shorter period of adrenal steroid administration did not restore the antagonist, the *in vitro* addition of hydrocortisone and other materials to the diaphragm system might not be adequate to produce insulin antagonism. The question also arises as to whether the insulin antagonist reported by Vallance-Owen and Lukens (12) in the depancreatized cat is the same material which we have found in the sera of patients in diabetic acidosis. The factor in the cat is stable to freezing and thawing and is in the globulin fraction. The antagonist in diabetic acidosis is not a lipoprotein and is found in the α_1-globulin fraction of the serum. Other chemical properties of the antagonist in the cat are not known. The antagonist in the cat was demonstrated using 1 milliunit of insulin per ml. of plasma, while in diabetic acidotic serum the antagonist was measured employing 200 milliunits of insulin per ml. of serum. Consequently, much smaller amounts could be detected in the cat serum. The use of much larger amounts of insulin in our test system could easily explain our failure to find insulin antagonist in all cases of diabetic acidosis examined. Only further experimental evidence will reveal whether these two factors are qualitatively similar.

SUMMARY

Further characterization of the insulin antagonist occurring in the sera of some patients in diabetic acidosis has been achieved. It was not inactivated by trypsin, but was destroyed by treatment with chymotrypsin. Upon starch block electrophoresis, it migrated with the α_1-globulin fraction of the serum proteins. However, there did not seem to be any increase in the total amount of α_1-globulins in the sera of four patients exhibiting insulin antagonist. Insulin antagonist serum did not exhibit any glucagon-like activity when in-

jected intravenously into fed rabbits, nor did the addition of glucagon to normal serum produce insulin antagonism. The addition of large amounts of monkey growth hormone, by itself and in combination with hydrocortisone, to normal serum did not inhibit the insulin effect on the rat hemidiaphragm. Serum obtained from a patient given human growth hormone in doses which caused some degree of insulin resistance was devoid of detectable insulin antagonist activity. The insulin antagonist was capable of inhibiting human insulin as well as beef-pork insulin in the diaphragm system.

Since the antagonist can oppose the action of human insulin it is suggested that it may be of etiologic significance in some cases of diabetes.

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STUDIES WITH THE SEROTONIN PRECURSOR, 5-HYDROXY-TRYPTOPHAN, IN EXPERIMENTAL ANIMALS AND MAN

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There is a widespread interest in the pharmacology of serotonin and its possible roles in normal physiology and pathologic states. Diverse actions of this substance in man are suggested by the syndrome of vasomotor disturbances, bronchoconstriction, intestinal hypermotility and valvular heart disease associated with hyperserotonemia due to secreting carcinoid tumors (1, 2). The potent stimulant action of serotonin on the estrous rat uterus *in vitro* (3) suggests that this substance might also affect uterine function *in vivo*. Suspicions concerning a role of serotonin in brain function have been supported by the demonstration that reserpine releases serotonin from the brain, as well as from other body depots (4). Although the importance of this agent is apparent, studies of its effects in the intact animal are limited by its rapid transformation to inactive metabolites after parenteral administration.

Recently, Udenfriend, Weissbach, and Bogdanski (5) demonstrated prolonged elevations of serotonin in various tissues following the administration of its precursor, 5-hydroxytryptophan (5HTP). It was found that 5HTP in a dosage of 30 to 60 mg. per Kg. in dogs produced marked effects which lasted several hours. These included tremors, ataxia, loss of contact plantar reflex, pupillary dilation, loss of light reflex, lacrimation, apparent blindness, salivation, hyperpnea, and tachycardia. These effects were considered to be similar to those produced by the hallucinogenic agent, lysergic acid diethylamide (LSD). It seemed worth while to ascertain whether chronic administration of 5HTP to animals would produce a sustained state of serotonin excess and whether alterations similar to the carcinoid syndrome, particularly endocardial fibrosis, would develop in animals treated in this manner. Also, the similarity in the actions of 5HTP and LSD in animals made it desirable to determine whether the former agent would induce central disturbances in man.

This paper consists of two parts. The first deals with observations on rats during chronic administration of 5HTP and includes studies on the effects of 5HTP on pregnant animals and their fetuses. The second part is devoted to the metabolism and pharmacological effects of 5HTP in man.

MATERIALS AND METHODS

The experimental animals were fed a standard commercial rat food[1] and allowed water *ad libitum*. Sprague-Dawley males weighing approximately 100 Gm. were used for the chronic studies. Pregnant rats were obtained from the Holtzman Rat Company; the duration of pregnancy was calculated from the time of observed mating.

The human subjects used in these studies were patients without evident renal or hepatic disease.

Platelet counts were done using direct phase contrast microscopy (6). For histologic studies tissues were fixed in 10 per cent buffered formalin. Sections were cut at a thickness of 6 microns and stained as follows: hematoxylin and eosin-y, azure and eosin, elastica (orcein) and Hale's iron stain for "mucopolysaccharides" (Longley's variant). Serotonin and 5-hydroxyindoleacetic acid (5HIAA) were measured by methods developed in this laboratory (7, 8).

Biochemical, pharmacologic and pathologic effects of 5HTP in rats. From previous observations made in this laboratory, it was considered likely that the administration of 5HTP at an appropriate dosage every 12 hours would maintain a state of chronic serotonin excess in animals. Accordingly, 5-hydroxy-DL-tryptophan was administered to rats in a dosage of 200 mg. per Kg. intraperitoneally. Twelve rats were injected every twelve hours for periods up to four months, and twelve others served as untreated controls. They were observed for changes in behavior, skin color, character of the feces and weight. Animals were randomly selected from each group and sacrificed at monthly intervals using pentobarbital intraperitoneally. The serotonin content of

[1] Purina rat pellets, Purina Mills, St. Louis, Missouri.

various tissues was determined and autopsy examinations were done. The following organs were examined microscopically: heart, liver, kidney, lung, skin, stomach, and cecum.

To obtain evidence that prolonged, elevated serotonin levels occurred in the chronic experimental animals, they were sacrificed at varying intervals of time after a given dose of 5HTP, and brain, ileum, kidney and liver were assayed. As shown in Table I the serotonin content of kidney and liver was markedly increased even 12 hours after 5HTP. In the brain, the serotonin returned to normal values within a few hours. The gut normally contains large amounts of serotonin, usually in the range of 7 to 15 μg. per Gm. The largest amount found in our chemically treated animals was 22 μg. per Gm. A value of this magnitude is occasionally encountered in control animals. Since the hearts were used entirely for histologic studies, separate experiments were carried out to demonstrate elevation of heart serotonin. In two rats, administration of 50 mg. per Kg. of 5HTP increased the levels of serotonin in the heart from less than 0.1 to over 2.5 μg. per Gm. in two hours. It is apparent that the administration of 200 mg. per Kg. of 5HTP every 12 hours is a satisfactory procedure for obtaining prolonged elevations of tissue serotonin levels *in vivo*.

Considering the large amounts of serotonin in their tissues, the animals which were on this regimen of 5HTP for periods of one to four months tolerated the agent remarkably well. The gain in weight of the experimental group was considerably less than that of the control group. The mean weights in grams at the beginning and end of the experiment in the treated and control groups were: 104 ± 10.2; 109 ± 9.6; 236 ± 17.4; and 375 ± 23.4, respectively. In spite of this, only one animal died during the study and this death was due to pneumonia. No distinct central effects were observed although within a few weeks the experimental animals appeared lethargic and relatively unresponsive to stimuli such as handling and injections. No significant gross or microscopic pathologic changes were found, even after four months of continuous treatment. Lesions of myocarditis, pyelonephritis and pneumonitis were observed but these were found to a comparable degree in both the control and experimental animals. Extensive study of the sections from the heart and skin showed no significant anatomic alterations. Platelet counts on three animals during the twelfth week of study ranged from

TABLE I

Tissue levels of serotonin after chronic administration of 5HTP

Hours after last injection	Weeks of treatment	Serotonin (*microgm./Gm.*)*		
		Brain	Kidney	Liver
0†	0	0.40	<0.10	<0.10
2	12	1.00	18.70	5.75
4–6	16	0.54	3.35	1.95
12	8	0.35	1.37	1.00

* Each value represents results obtained on homogenate of two separate rats.

† Controls.

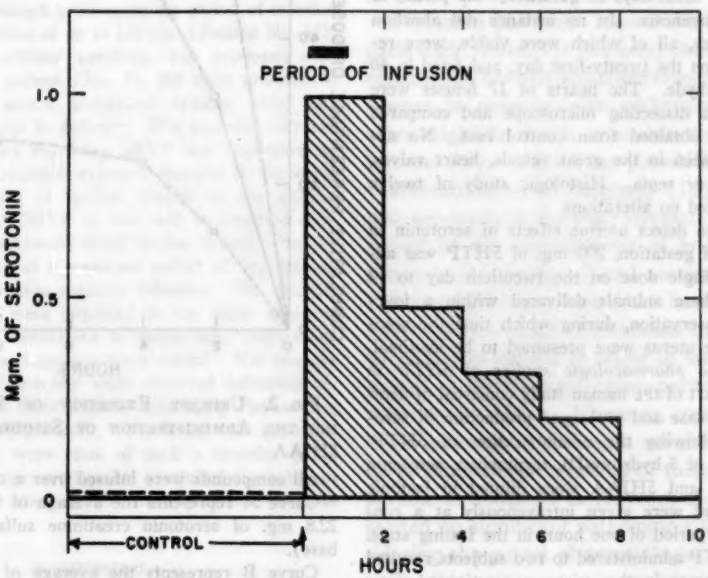

FIG. 1. URINARY EXCRETION OF SEROTONIN (MG. PER TWO HOURS) IN MAN AFTER INTRAVENOUS ADMINISTRATION OF 50 MG. OF 5HTP

Values are the average of two studies.

TABLE II

Effect of 5HTP on fetal and uterine serotonin*

Tissue	Serotonin (*microgm./Gm.*)†	
	Controls	2 hours after 5HTP
Fetus	<0.10	5.0
Uterus	<0.10	2.5

* Dosage, 200 mg. per Kg.

† Each value represents results obtained on homogenate of two separate rats.

600,000 to 990,000 per cubic mm. These were comparable to values obtained on control animals.

The most obvious effects of 5HTP were on the gastrointestinal tract and on cutaneous vasculature. The first consisted of marked diarrhea which became apparent within one week and continued for about two weeks, after which it persisted in a milder form. Secondly, within a few weeks a bluish discoloration was observed on the skin of the nose, ears, feet, and tail. This "cyanotic" appearance, indicative of a marked vasomotor disturbance, was most evident four to six hours after each injection, but was constantly present on the tails.

As shown in Table II, administration of 5HTP to pregnant rats resulted in a marked elevation in the levels of serotonin in the uterus and fetus. The following experiments were designed to investigate the effects of serotonin on uterine function during pregnancy and on cardiogenesis *in utero*. Twelve pregnant rats were given 200 mg. per Kg. of 5HTP twice a day on the seventh, eighth, ninth, and tenth days of gestation,[2] the period of most active cardiogenesis. In no instance did abortion occur. The fetuses, all of which were viable, were removed surgically on the twenty-first day, and fixed in 40 per cent formaldehyde. The hearts of 17 fetuses were examined under a dissecting microscope and compared with fetal hearts obtained from control rats. No abnormalities were seen in the great vessels, heart valves, chordae tendinae or septa. Histologic study of twelve fetal hearts revealed no alterations.

In an attempt to detect uterine effects of serotonin in the later stages of gestation, 200 mg. of 5HTP was administered in a single dose on the twentieth day to 12 rats. None of these animals delivered within a four-hour period of observation, during which time the serotonin levels in the uterus were presumed to be maximal.

Biochemical and pharmacologic studies of 5HTP in man. The first part of the human study consisted of demonstrating an increase and prolonged production of serotonin in man following the administration of 5HTP. Various amounts of 5-hydroxy-DL-tryptophan, serotonin creatinine sulfate and 5HIAA were diluted in isotonic NaCl solution and were given intravenously at a constant rate over a period of one hour, in the fasting state. Fifty mg. of 5HTP administered to two subjects resulted in a marked increase in the urinary excretion of serotonin for several hours (Figure 1). The percentages of the administered dose of 5HTP represented by urinary serotonin in the two subjects were 4 and 7 per cent, respectively. There was no detectable increase in urinary serotonin following the administration of 10 mg. of serotonin itself. Thus, in man as in experimental animals, it would appear that 5HTP produces a prolonged increase of serotonin in the body. No attempt was made to measure the effect of 5HTP on circulating serotonin since previous studies with animals indicated that at the dosage used in these experiments, the increase in blood serotonin would be barely perceptible. Further information concerning the fate of serotonin, its precursor (5HTP), and its metabolite (5HIAA) in man, was obtained by measurement of the urinary excretion of 5HIAA following the administration of each of the three substances. As shown in Figure 2, the average cumulative percentage of each substance excreted as 5HIAA was found to be: serotonin, 77 per cent (range, 65 to 92); 5HIAA, 55 per cent (range, 40 to 70); and 5HTP, 28 per cent (range, 14 to 35).

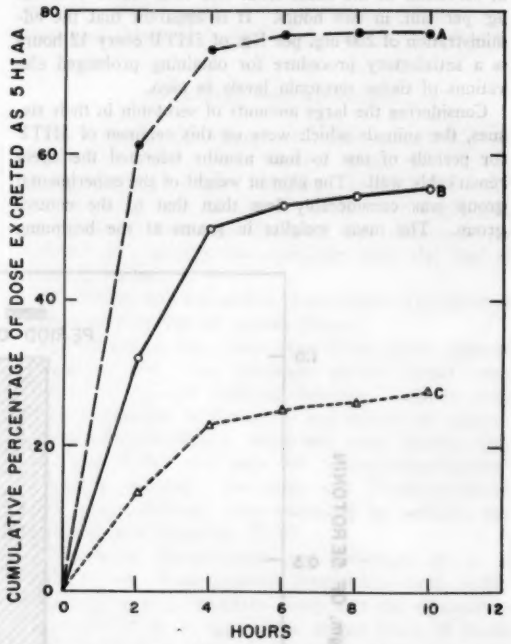

FIG. 2. URINARY EXCRETION OF 5HIAA FOLLOWING THE ADMINISTRATION OF SEROTONIN, 5HTP, AND 5HIAA

All compounds were infused over a one-hour period.

Curve A represents the average of three infusions of 22.8 mg. of serotonin creatinine sulfate (10 mg. free base).

Curve B represents the average of two infusions of 10.8 mg. of 5HIAA.

Curve C represents the average of five infusions in which the dose of 5HTP varied from 12.5 to 100 mg.

[2] Period of gestation in the rat is 21 days.

TABLE III

Effects of 5HTP in human subjects

Patient No.	Total dose (mg.)	Period of infusion	Effects
1	10.4	10 sec.	Moderate nausea beginning in 20 min., lasting an hour
2	25.0	30 min.	Mild intermittent nausea and abdominal cramping
3	25.0	10 sec.	Mild nausea for 10 minutes
	25.0	60 min.	Mild nausea for two hours after end of infusion
	34.0	51 min.	Severe nausea and frequent retching for two hours after end of infusion
4	20.0	10 sec.	Mild nausea for 5 min.
5	22.5	10 sec.	Moderate nausea for 10 min.
	25.0	5 min.	Moderate nausea, abdominal cramps and urge to defecate lasting 10 min.
	29.0	10 min.	Moderate nausea, abdominal cramps and urge to defecate lasting 1½ hours
	41.0	60 min.	Severe nausea, abdominal cramps, urge to defecate, and borborygmi lasting 2 hours
	50.0	60 min.	Severe nausea, retching, cramps, flatus, urge to defecate, and borborygmi lasting 2 hours
6	25.0	60 min.	Mild nausea for 5 min.
7	25.0	60 min.	Mild nausea for 5 min.
8	27.0	40 min.	Moderate nausea, occasional retching, and increased bowel sounds lasting 5 hours
9	30.0	10 sec.	Mild nausea for 10 min.
10	50.0	60 min.	Mild nausea, cramping, increased bowel sounds, and projectile vomiting for 10 min.
11	120.0	60 min.	Mild nausea for 30 min.

The pharmacologic effects observed following 5HTP administration in 11 human subjects are summarized in Table III. Initially, infusions were given over a period of 10 seconds and in each case nausea developed after a latent period of 15 to 20 minutes with a dosage of 10 to 30 mg. (Patients Nos. 1, 3, 4, 5, 9). Subsequent infusions were given over longer periods of time (5 to 60 minutes) in the hope of obviating the nausea and thereby administering a dose sufficiently large to produce mental disturbances. Although prolonging the period of infusion enabled administration of up to 120 mg. (Patient No. 11), nausea, with or without vomiting, was produced consistently. In one patient (No. 5), the most pronounced symptoms were severe abdominal cramps with borborygmi and an urge to defecate. The apparent increase in intestinal motility following 5HTP was confirmed by recordings of intraluminal pressure changes in the small bowel. The results of further studies on the gastrointestinal effects of 5HTP in man will be reported elsewhere (9). An untoward effect in one patient (No. 3) was the occurrence of a transient period of hypotension following each of three separate infusions. No changes in blood pressure were observed in the other subjects, however, and no alterations in pupil size, respiratory rate and electrocardiograms were noted. No obvious changes in mental function were observed following infusion of 5HTP in these patients (including Patient No. 10, who was schizophrenic). However, the gastrointestinal symptoms were often of such a severity as to invalidate psychologic testing and precluded the achievement of dosages which might have produced definite central effects.

DISCUSSION

It is apparent from these studies that a prolonged increase in the production of endogenous serotonin can be achieved in both man and experimental animals by the administration of 5HTP. Furthermore, biochemical adaptation does not develop since, in the chronic studies, a state of continuous serotonin excess was found in rat tissues. The chronic studies in rats were also of importance in providing information concerning the toxicity of 5HTP prior to studies in man.

Serotonin has been implicated in a variety of pathologic states. It has been considered that the endocardial fibrosis and concomitant valvular heart disease in patients with malignant carcinoid tumors might be due to serotonin secreted by these tumors (2). Also, the possibility occurred to Waldenström and Ljungberg (10) and ourselves that serotonin in the fetal circulation might be involved in the pathogenesis of endocardial fibroelastosis of infancy. Renal cortical necrosis (11, 12) and thrombocytosis (13) have been reported following injections of serotonin in rats. The renal lesion is known to occur in such clinical conditions as *abruptio placentae*, peritonitis and acute pancreatitis (14). However, rats subjected to huge amounts of serotonin administered through its precursor over a period of their life span comparable to about 10 to 15 years of human life exhibited no significant pathologic alterations. Considering the potent pharmacologic actions of serotonin, this was a most surprising result but was reassuring in terms of administering the compound to human subjects. Although limited supplies of

5HTP prevented giving the compound throughout the entire period of gestation in the rat, fetal hearts subjected to excess serotonin *in utero* during the period of cardiogenesis showed no abnormalities.

Since the rat uterus in estrous is sensitive to serotonin *in vitro*, it was also suspected that 5HTP might be a powerful abortifacient. In spite of the fact that high levels of serotonin were attained in the uterine muscle of pregnant rats, abortion did not occur. It would appear that elevation of uterine serotonin *in vivo* causes no marked alteration in uterine activity. Although it has been pointed out that much of the serotonin found in tissues a few hours after administration of 5HTP exists in a bound, inactive form, it was also shown that within the first two hours after 5HTP administration high levels of free, pharmacologically active serotonin are found in the plasma (5).

The cutaneous vasomotor disturbances and diarrhea in 5HTP-treated rats are consistent with known actions of serotonin and are similar to symptoms which are seen in patients with malignant carcinoid. In patients given 5HTP, nausea and increased intestinal motility were the only consistent effects, further demonstrating the sensitivity of the human gut to serotonin. Since the gastrointestinal tract contains most of the serotonin in the body, these findings may signify a role of serotonin in gastrointestinal motility.

Although no mental alterations were noted in the human subjects, the dosage of 5HTP was limited by the intestinal effects. Since 20 to 60 mg. per Kg. of 5HTP were required to produce central disturbances in dogs and cats (15) it is not surprising that the amounts used in our studies (0.1 to 2.0 mg. per Kg.) had no central effect. Further attempts should be made to infuse larger amounts of this substance in man. In this regard, it may be possible to block the peripheral effects of 5HTP pharmacologically and thereby achieve dosages sufficiently high to produce central effects in man. Such studies may help clarify recent speculation concerning the effects of serotonin in the brain, which has been gathered exclusively from studies with drugs structurally related to serotonin.

SUMMARY

1. The chronic administration of the serotonin precursor, 5-hydroxytryptophan (5HTP), has been shown to produce sustained elevations of serotonin levels in rats. Rats so treated for periods up to 120 days exhibited diarrhea and cutaneous vasomotor disturbances but showed no discernible anatomic alteration on histologic examination.

2. Prolonged increases in tissue serotonin in man also were achieved with 5HTP. The predominant pharmacologic effect of 5HTP in man was stimulation of the gastrointestinal tract which precluded the attainment of a dosage sufficiently high to produce central effects comparable to those seen in experimental animals.

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QUANTITATIVE STUDIES ON HUMAN URINARY METABOLITES OF TRYPTOPHAN AS AFFECTED BY ISONIAZID AND DEOXYPYRIDOXINE [1]

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Peripheral neuropathy has been observed as a complication of the treatment of tuberculosis with isonicotinic acid hydrazide (isoniazid) (1, 2), especially when large doses have been employed (3, 4). This complication of isoniazid therapy has been largely eliminated by the simultaneous administration of pyridoxine (3, 5).

In pyridoxine deficiency, rats (6) and humans (7) have been found to excrete abnormally large amounts of the tryptophan metabolite xanthurenic acid. The administration of the pyridoxine antagonist, deoxypyridoxine, to humans (8) or rats (9) has been shown to produce a severe disturbance of tryptophan metabolism in these species as manifested by the excretion of large amounts of xanthurenic acid following the administration of a "loading" dose of tryptophan. These observations have prompted studies of the urinary excretion of xanthurenic acid as an index of the antipyridoxine activity of isoniazid. Studies with humans (3) and rats (10) ingesting isoniazid have revealed an increased excretion of xanthurenic acid after ingestion of tryptophan. Rosen (11) reported no increase in xanthurenic acid excretion when isoniazid was administered to pyridoxine-deficient rats, even though the rats showed other signs of pyridoxine deficiency. In the studies of Biehl and Vilter (3) the administration of large doses of isoniazid resulted in a considerable increase in urinary excretion of vitamin B_6, about a 40 per cent incidence of clinical evidence for pyridoxine deficiency, but an irregular elevation of xanthurenic acid excretion following a tryptophan load. Thus it appeared that the clinical evidence for pyridoxine deficiency in rats or humans given isoniazid was more striking than the xanthurenic acid excretion studies would suggest (3, 11).

The present studies were undertaken in an effort to determine whether or not the simultaneous quantitative measurement of several urinary metabolites of tryptophan would reveal additional evidence of a disturbance in the metabolism of this essential amino acid in patients ingesting isoniazid. The extensive paper chromatographic studies of Dalgliesh (12) revealed that pyridoxine-deficient rats excreted not only xanthurenic acid, but large amounts of several other intermediary metabolites of tryptophan. Methods have been developed for the quantitative determination of several of these intermediary metabolites of tryptophan in urine (13–15). The application of these methods in quantitative studies of tryptophan metabolism in normal human subjects has been reported (14, 16).

The present detailed studies on tryptophan metabolism were made repeatedly over a period of several weeks during which time the dosage of isoniazid was increased until abnormal tryptophan metabolism was observed. Once the metabolic pattern became abnormal, pyridoxine and three other members of the vitamin B-complex, each of which has been suggested to play some role in tryptophan metabolism, were administered in an attempt to determine which of these vitamins might be required to overcome the effects of isoniazid on this metabolic pathway. A preliminary report of these studies has been made (17).

EXPERIMENTAL

Analytical procedures. The methods for the determinations of urinary kynurenic acid, xanthurenic acid, *N*-methyl-2-pyridone-5-carboxamide (pyridone), anthranilic acid glucuronide, *N*-α-acetylkynurenine, kynurenine, o-aminohippuric acid, and 3-hydroxykynurenine were those previously described (13–15). Paper chromatog-

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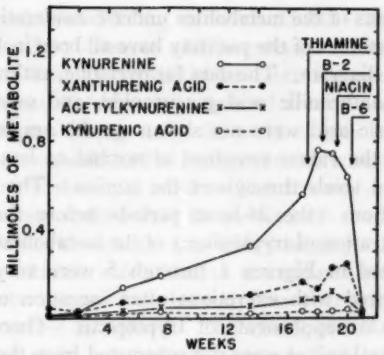

FIG. 1. MILLIMOLES OF METABOLITES EXCRETED IN THE 24-HOUR PERIOD FOLLOWING INGESTION OF TRYPTOPHAN

Subject 1 (weight, 77 Kg.) was receiving 300 mg. of isoniazid daily at the start of the experiment. The dose was increased to 600 mg. after two weeks and was given at a level of 750 mg. daily from the start of the sixth week until the end of the experiment. B-2 = riboflavin; B-6 = pyridoxine hydrochloride.

raphy (14, 18) was utilized to verify the quantitative determinations of aromatic amines in every urine studied.

Subjects. The studies were conducted on six male patients with tuberculosis from the Veterans Administration Hospital, but the detailed data presented were obtained from three of these subjects. Subject 2 was used for three of the studies and the others were used for one study each. Subjects 1 and 2 were Negroes, and subject 3 was white. The subjects all had pulmonary tuberculosis but were in a stationary phase of their disease. The subjects were ambulant and afebrile. Genitourinary tuberculosis was specificially excluded.

Each patient was thoroughly instructed concerning the proposed experiments. At weekly intervals (except

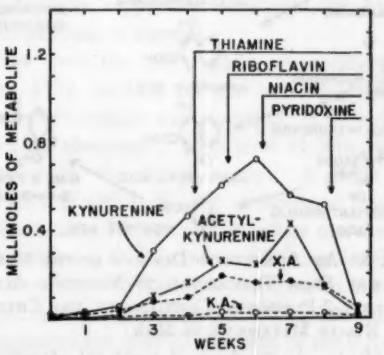

FIG. 2. MILLIMOLES OF METABOLITES EXCRETED IN THE 24-HOUR PERIOD FOLLOWING INGESTION OF TRYPTOPHAN

Subject 2 (weight, 57 Kg.) was not on therapy at the start of the experiment. Isoniazid was started at a level of 300 mg. daily after the initial study and increased to 450, 600, 750 mg. daily after the first, second, and third week, respectively, and continued throughout the study at a level of 750 mg. daily. X. A. = xanthurenic acid; K. A. = kynurenic acid.

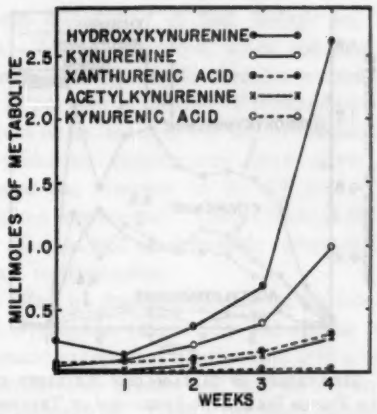

FIG. 3. MILLIMOLES OF METABOLITES EXCRETED IN THE 24-HOUR PERIOD FOLLOWING INGESTION OF TRYPTOPHAN

Subject 3 (weight, 51 Kg.) was started on 300 mg. of isoniazid daily after the initial, zero-time study and the dose was increased to 450, 600, and 900 mg. daily after the first, second, and third week, respectively. This study was discontinued because the patient left the hospital after the fourth week. Note that the scale of this graph is different from Figures 1 and 2.

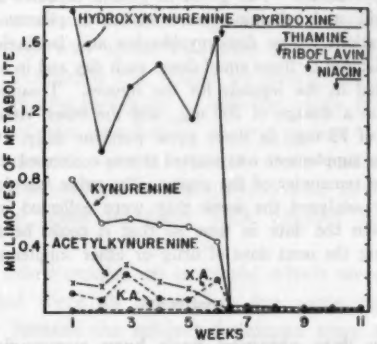

FIG. 4. MILLIMOLES OF METABOLITES EXCRETED IN THE 24-HOUR PERIOD FOLLOWING INGESTION OF TRYPTOPHAN

Subject 2 was given 900 mg. of isoniazid daily starting at zero time, but had not received any chemotherapy for 18 weeks prior to the time this study was started. No pre-treatment study was done, but the patient had a normal pre-treatment response to the 2 Gm. of tryptophan in the other experiments in which he participated (Figures 2 and 5). X. A. = xanthurenic acid; K. A. = kynurenic acid.

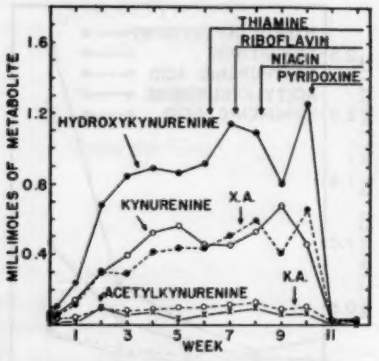

FIG. 5. MILLIMOLES OF METABOLITES EXCRETED IN THE 24-HOUR PERIOD FOLLOWING INGESTION OF TRYPTOPHAN

Deoxypyridoxine was given to subject 2 at a level of 75, 150, 225, 300, and 375 mg. daily starting after 0, 1, 2, 3, and 5 weeks, respectively, and the latter dose was continued throughout the study. X. A. = xanthurenic acid; K. A. = kynurenic acid.

for Figure 1) the following procedure was carried out: a 24-hour urine collection (in amber bottles under toluene) was started at 7 a.m. on Monday, and the subject ingested 2.0 Gm. (9.8 mM) of L-tryptophan at 7 a.m. on Tuesday. A second 24-hour urine collection was completed by 7 a.m. on Wednesday and all changes in medication were made at this time.

The tryptophan was administered as a single dose in four 0.5-Gm. gelatin capsules (Figures 1 and 2) or as four 0.5-Gm. tablets[3] (Figures 3 through 5). The deoxypyridoxine[4] was given in gelatin capsules and the isoniazid and vitamins were standard pharmaceutical preparations. The deoxypyridoxine and isoniazid were administered in three equal doses each day and in amounts indicated in the legends for the figures. Thiamine was given at a dosage of 300 mg., and the other vitamins at levels of 75 mg., in three equal portions daily. Once a vitamin supplement was started it was continued throughout the remainder of the study. The urine samples were always analyzed the week they were collected in order to obtain the data in time so that it could be used in selecting the next dose of drug or other supplement.

RESULTS

The data obtained have been summarized in Figures 1 through 5. An abbreviated outline of the metabolic pathway from tryptophan to nicotinic acid has been presented in Figure 6. The positions of the metabolites under consideration in these studies of the pathway have all been included in the diagram. The data for pyridone, anthranilic acid, anthranilic acid glucuronide, and o-aminohippuric acid were not shown on the graphs because the values remained at normal or less than normal levels throughout the studies. The basal excretions (the 24-hour periods before the administration of tryptophan) of the metabolites represented in Figures 1 through 5 were very low compared with excretions after ingestion of the 9.8 mM supplements of tryptophan. Therefore, the basal values were not subtracted from the data presented in the graphs. Thus the data in Figures 1 through 5 represent the millimoles of metabolites excreted in the 24-hour period following ingestion of the 9.8 mM of tryptophan (Figure 1). Unless otherwise indicated all values below refer to the 24 hours after tryptophan.

Although subject 1 (Figure 1) was receiving 300 mg. of isoniazid daily at the start of the experiment, the urinary excretion of metabolites was normal for the first two studies. After three weeks

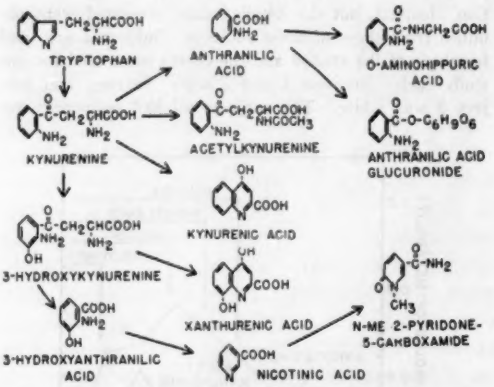

FIG. 6. AN ABBREVIATED DIAGRAM OF THE METABOLIC PATHWAY FROM TRYPTOPHAN TO NICOTINIC ACID AND *N*-METHYL-2-PYRIDONE-5-CARBOXAMIDE, THE CHIEF URINARY NIACIN METABOLITE IN MAN

Pyridoxine, in the form of pyridoxal phosphate, has been reported to function in the conversion of: 1) kynurenine to anthranilic acid (36); 2) 3-hydroxykynurenine to 3-hydroxyanthranilic acid (37); 3) kynurenine to kynurenic acid (37); and 4) 3-hydroxykynurenine to xanthurenic acid (37). Niacin in the form of triphosphopyridine nucleotide was required for the hydroxylation of kynurenine to hydroxykynurenine (18) and riboflavin also has been suggested to play a role in this reaction (24–26).

[3] The tryptophan was compressed into 0.5-Gm. tablets for these studies by Dr. Rodney P. Gwinn and his associates at Abbott Laboratories, North Chicago, Illinois.

[4] The deoxypyridoxine was generously supplied by Dr. Elmer Severinghaus of Hoffmann-La Roche, Inc., Nutley, New Jersey.

of ingestion of 600 mg. of isoniazid daily, the excretion of kynurenine and acetylkynurenine were increased to abnormal levels after ingestion of tryptophan. After the isoniazid intake was increased to 750 mg. daily the yield of kynurenine, xanthurenic acid and acetylkynurenine rose to increasingly abnormal levels. The vitamin supplements were started after the seventeenth week and an additional vitamin was added weekly. After the pyridoxine was added to the vitamin supplement the response to the test dose of tryptophan was restored to normal. The effects of the other three vitamin supplements were of doubtful significance, though there was a slight drop in kynurenine excretion when the niacin supplement was started.

The second experiment (Figure 2) was essentially a repetition of the first study and gave comparable results. Subject 2 did not receive any isoniazid when the initial study was done. This subject usually excreted more acetylkynurenine than xanthurenic acid, while the opposite was true with subject 1. The subject was kept on supplements of thiamine, riboflavin, and nicotinamide for two weeks before the pyridoxine was started, since the results obtained with the first subject suggested that a slight decrease in kynurenine occurred after niacin was started. Again the addition of niacin slightly decreased the yield of kynurenine. The pyridoxine supplements restored the tryptophan metabolism to normal.

By the time the next study was undertaken (Figure 3) a method for the determination of 3-hydroxykynurenine was available. This subject excreted an abnormally high level of 3-hydroxykynurenine in the initial study. A daily administration of 450 to 600 mg. of isoniazid resulted in a severe disturbance of tryptophan metabolism and the last study at four weeks (900 mg. isoniazid daily) revealed a remarkable increase in the excretion of 3-hydroxykynurenine. This one metabolite accounted for over 25 per cent of the 9.8 mM supplement of tryptophan, and the kynurenine accounted for over 10 per cent of the amino acid given. Acetylkynurenine and xanthurenic acid were excreted in approximately equal quantities, and as in all studies on isoniazid the kynurenic acid excretion was less than normal.

In the next study (Figure 4) the subject was started on 900 mg. of isoniazid daily at zero time and was maintained at that dosage for eleven weeks. Within one week, when the first study was made, the hydroxykynurenine accounted for about 17 per cent of the dose of tryptophan. After six weeks on an intake of 900 mg. of isoniazid daily the pyridoxine supplements were started, and thereafter the response to the 9.8 mM dose of tryptophan was normal. The addition of the other three vitamins had no detectable influence on the response to tryptophan.

The effect on deoxypyridoxine on the fate of ingested tryptophan (Figure 5) was similar to that of isoniazid except that xanthurenic acid excretion was considerably greater and was almost equal to that of kynurenine. Unlike the observations during isoniazid treatment the administration of deoxypyridoxine was accompanied by abnormally high kynurenic acid excretion. Again, although the addition of thiamine, riboflavin and niacin supplements had little, if any, effect on the metabolism of the test dose of tryptophan, pyridoxine restored the urinary excretion of metabolites to normal.

Another subject was started on deoxypyridoxine (subject 3) at a level of 150 mg. daily. Within one week the response to tryptophan ingestion was similar to that in Figure 5 at the same dosage of deoxypyridoxine (2 weeks). The first supply of deoxypyridoxine was exhausted at this point and a new batch was used. The second week (end of one week on the new supply) the response to tryptophan was about half that of the previous week. Although the intake of deoxypyridoxine was gradually increased to 450 mg. daily the excretion of the metabolites of tryptophan fell to almost normal by the seventh week, indicating that the second lot of deoxypyridoxine probably contained some substance with vitamin B_6 activity.[5]

The other studies on isoniazid which were not presented were all incomplete for some reason, usually because the subject developed some additional illness. The incomplete results were in general agreement with the data presented (Figures 1 through 4).

None of the subjects experienced any symp-

[5] Hoffmann-La Roche, Inc., reported that samples of the two lots of deoxypyridoxine have now been tested microbiologically for antipyridoxine activity, and the first batch of drug had about 20 times the activity of the second.

toms that could be attributed to the ingestion of either isoniazid or deoxypyridoxine.

Although, as stated previously, the basal excretions of the various metabolites (the 24-hour periods prior to tryptophan supplementation) were of insignificant quantities compared with the excretion following ingestion of tryptophan, they were altered somewhat by isoniazid or deoxypyridoxine. Thus, kynurenic acid was usually excreted at basal levels of 7 to 10 μM daily and the ingestion of isoniazid or deoxypyridoxine decreased these levels to 3 to 8 μM per day. Basal values for kynurenine excretion ranged from 8 to 12 μM daily but invariably increased to 35 to 45 μM daily at the times when the response to the tryptophan load yielded quantities of 700 to 1,000 μM of this metabolite. The pre- and post-tryptophan values for urinary o-aminohippuric acid and anthranilic acid glucuronide were very similar to those observed in studies on normal human subjects (14), and the volatile amine from the acetylkynurenine fraction (14) was usually comparable to the values found on direct diazotization and coupling of this fraction, which indicated that little, if any, free anthranilic acid was excreted.

Although the pyridone excretion usually remains elevated for several days after the ingestion of tryptophan (14, 16) or niacin (19) the amount excreted the first day provides some indication of niacin formation from tryptophan. In general, the subjects excreted less than the expected amounts of pyridone following ingestion of tryptophan. Subject 1, however, invariably excreted more than the expected amounts of pyridone following ingestion of tryptophan, so that in these studies no conclusions could be made concerning the effects of isoniazid on the conversion of tryptophan to niacin. When the nicotinamide supplements were given to these subjects the pyridone excretion increased to very high levels, which suggested that isoniazid and deoxypyridoxine had little, if any, effect on the conversion of niacin to the pyridone.

DISCUSSION

In these studies the subjects were maintained on a general hospital diet and no attempt was made to regulate the food intake. The subjects maintained their weights or gained slightly during the experiments. Previous work has indicated that the urinary excretion of these metabolites of tryptophan (except for the pyridone) was about the same on self-selected or constant dietary intake (16). The magnitude of the disturbance of tryptophan metabolism observed in the present studies makes it appear unlikely that a carefully regulated diet would have contributed much to the results. However, a larger dietary intake of pyridoxine probably would have made it necessary to administer higher doses of isoniazid and deoxypyridoxine to achieve effects similar to those observed.

All of the subjects used in these studies had essentially normal tryptophan metabolism prior to the ingestion of isoniazid or deoxypyridoxine except for subject 3 who, for unknown reasons, excreted an abnormal amount of 3-hydroxykynurenine. Abnormal tryptophan metabolism has been found in patients with tuberculosis (20, 21). However, Dalgliesh and Tekman (22) reported that large quantities of kynurenine and hydroxykynurenine were excreted by patients, including those with tuberculosis, only if there was an elevation of body temperature. The fact that the subjects used in the present studies did not have an elevation of the body temperature and had normal tryptophan metabolism in the absence of the drugs corroborates these findings.

Biehl and Vilter (3) reported that not all tuberculous patients receiving isoniazid therapy excreted abnormal quantities of xanthurenic acid after tryptophan loading. The present studies, however, leave little doubt that large doses of isoniazid produced a profound disturbance in the metabolism of a test dose of tryptophan. Normal subjects excreted an additional 3 to 5 μM of acetylkynurenine and 15 to 30 μM of kynurenine during the first 24 hours after ingestion of 9.8 mM of L-tryptophan (14, 16). In subjects ingesting isoniazid, however, the yield of acetylkynurenine reached 20 to 55 times, and of kynurenine 20 to 30 times, these quantities. Xanthurenic acid excretion was not nearly as useful as the excretion of kynurenine or hydroxykynurenine in detecting inability to metabolize tryptophan in a normal manner, and kynurenic acid excretion was usually normal or less than normal in these subjects. There appear to be some clinical conditions where the urinary excretion of xanthurenic acid may be normal or less than normal after ingestion of a

"loading dose" of tryptophan, while kynurenic acid, kynurenine, and other metabolites may be found in very abnormally high levels (23). Thus the determination of a single urinary metabolite of tryptophan cannot be relied upon as an indication of abnormal tryptophan metabolism.

Pyridoxine appeared to correct the metabolism of tryptophan without any definite evidence for the need of additional thiamine, riboflavin, or niacin. Niacin in the form of triphosphopyridinenucleotide has been shown to be required for the hydroxylation of L-kynurenine to 3-hydroxy-L-kynurenine (18). There was some evidence that niacin administration slightly decreased the excretion of kynurenine, but since hydroxykynurenine was not determined in these studies (Figures 1 and 2) it was not known whether or not these observations indicated increased hydroxylation of kynurenine. Considerable hydroxykynurenine was excreted by the subjects ingesting the largest doses of isoniazid (Figures 3 and 4) which would indicate that in this one reaction the isoniazid failed to reveal significant antiniacin activity.

Riboflavin supplements were administered to these subjects because it has been reported (24–27) that this vitamin may be involved in the metabolism of tryptophan. Dalgliesh (28) found that rats deficient in both pyridoxine and thiamine failed to excrete the urinary metabolites of tryptophan which were detected in pyridoxine deficiency, and suggested that thiamine may be involved in the formation of formylkynurenine from tryptophan. In the present experiments there was no demonstrable effect of riboflavin supplements on tryptophan metabolism. A slight increase in kynurenine excretion occurred after the thiamine supplements were started, but this was observed under conditions where the kynurenine excretion was increasing, probably because of the administration of the drugs.

In previous studies (16) conducted in a manner similar to those reported here, but with normal subjects on a constant dietary regimen, it was found that the increase in urinary tryptophan metabolites after supplementation with 9.8 mM of the amino acid would account for about 2 per cent of the administered tryptophan. Slightly less than half of the tryptophan was accounted for as the pyridone metabolite of niacin. Such results might be interpreted to indicate that very little tryptophan enters the kynurenine pathway under normal conditions. In the present experiments as much as 40 per cent of the 9.8 mM dose of tryptophan was accounted for in the urine in the form of kynurenine and its metabolites. Thus, subjects ingesting isoniazid either converted more than normal amounts of tryptophan to kynurenine and related substances, or they were unable to convert these intermediates to the usual end products at a normal rate.

The enzyme system tryptophan peroxidase, which initiates the conversion of tryptophan to kynurenine, has been shown to be adaptive (29). Administration of substances like kynurenine, histamine, phenylalanine, cortisone, and especially tryptophan itself have been found to increase the activity of tryptophan peroxidase in rat liver (29). Thus it may be possible that patients ingesting isoniazid convert more than the normal quantity of tryptophan to kynurenine. However, since there is so much evidence to indicate that isoniazid has an antagonistic effect on pyridoxine in man (3, 5) and the metabolites found in large quantities in the urine in the present studies were identical with those detected in the urine of pyridoxine-deficient rats (12), it appears reasonable to explain the present findings in terms of incomplete metabolism of the kynurenine produced. Dalgliesh and Tabechian (30) have recently discussed the metabolic fate of tryptophan and have suggested that considerable tryptophan may normally enter this pathway and may be converted to 3-hydroxyanthranilic acid and then to non-aromatic products. This conclusion was reached by eliminating other metabolites of kynurenine, such as kynurenic and xanthurenic acids, as likely precursors of unknown major metabolites because they appeared to be relatively inert from a metabolic standpoint, while 3-hydroxyanthranilic acid has been shown to be metabolized rapidly (31–33). Although it is now known that kynurenic acid and xanthurenic acid cannot be regarded as metabolically inert, since both have been shown to be dehydroxylated in the 4-position (34, 35), there is little evidence to suggest that these quinoline derivatives are excreted in quantities adequate to account for the majority of supplements of L-tryptophan. The results of the present study, therefore, cannot be regarded as in disagreement with the explanation offered by Dalgliesh and Tabechian (30) for the

normal metabolic fate of the major part of a tryptophan supplement.

The differences in the patterns of the urinary metabolites produced by isoniazid and deoxypyridoxine were striking. Xanthurenic and kynurenic acids were both excreted in abnormally large quantities during ingestion of deoxypyridoxine, while less than normal amounts of kynurenic acid and only moderately increased quantities of xanthurenic acid were excreted under the influence of isoniazid. Since kynurenine and hydroxykynurenine were produced in large amounts in the presence of each drug it would appear that *in vivo* isoniazid was a better inhibitor of kynurenine transaminase than deoxypyridoxine.

SUMMARY

The influence of isoniazid and deoxypyridoxine on the urinary excretion of tryptophan metabolites by tuberculous patients was studied. The subjects all had essentially normal tryptophan metabolism prior to treatment with these drugs.

During treatment with isoniazid or deoxypyridoxine, 3-hydroxykynurenine was excreted in the urine in quantities adequate to account for from 10 to 25 per cent of a 9.8 mM (2 Gm.) supplement of L-tryptophan. Kynurenine was often excreted in quantities equivalent to 5 to 10 per cent of the tryptophan administered, while *N*-α-acetylkynurenine and xanthurenic acid were also excreted in abnormally large amounts. The excretion of kynurenic acid was slightly less than normal during isoniazid treatment and abnormally high when deoxypyridoxine was administered. Normal or slightly less than normal amounts of o-aminohippuric acid, anthranilic acid glucuronide, anthranilic acid and *N*-methyl-2-pyridone-5-carboxamide were found in the urine after ingestion of tryptophan during treatment with either drug.

Pyridoxine administration resulted in a return to normal tryptophan metabolism even when the ingestion of isoniazid or deoxypyridoxine was continued. Supplemental thiamine, riboflavin, or niacin had little, if any, effect on the abnormal tryptophan metabolism produced by these drugs.

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STUDIES IN CUSHING'S SYNDROME. II. ADRENAL WEIGHT-MAINTAINING ACTIVITY IN THE PLASMA OF PATIENTS WITH CUSHING'S SYNDROME [1]

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Patients with Cushing's syndrome due to bilateral adrenal cortical hyperplasia have been reported by several investigators to show an exaggerated plasma 17-hydroxycorticosteroid response to administered ACTH (1–3). Although pituitary corticotropin has been suspected of playing an etiologic role in this disorder, previous workers have generally failed to detect elevated levels of ACTH in the blood of patients with Cushing's syndrome (4–7). Furthermore, the prolonged administration of ACTH to normal subjects has not been observed to cause adrenal cortical hyperresponse comparable to that found in bilateral adrenal hyperplasia (8, 9).

In search of an explanation for this adrenal hyper-responsiveness, the possibility has been investigated of another type of corticotropic activity, different from the usual ACTH (10–12). The purpose of this communication is to present data which indicate the occurrence of adrenal weight-maintaining activity in the plasma of patients with Cushing's syndrome associated with adrenal hyperplasia.

METHODS AND MATERIALS

Plasma for assay was obtained from several groups of patients (*vide infra*) in the following manner. Samples of 100 to 200 ml. of blood were collected in iced tubes with heparin added, plasma was quickly separated in a cold centrifuge, and then frozen and stored at −20° C. in small tubes containing sufficient material (6 to 9 ml.) for a single day's injection. In three instances, plasma was lyophilized and reconstituted with distilled water to the original volume.

The assay method used was a modification of that devised by Simpson, Evans, and Li in which corticotropic activity was measured by the ability of a substance to prevent adrenal atrophy in the rat following hypophysectomy (13). Male Sprague-Dawley rats were hypophysectomized at 30 days of age. Injections of the plasma to be assayed were begun 24 hours later. The dosage of plasma was 0.3 ml. given subcutaneously three times a day for nine days, making a total dose of 8 to 9 ml. per rat. Plasma from each individual patient (or in some instances, from each plasma pool, *vide infra*) was administered to a group of six to nine rats. The control for each group of experimental rats was a group of six to ten animals of exactly the same age, hypophysectomized on the same day, and maintained identically throughout each experimental period, except that no injections were given. As an additional control for patients' plasma, groups of hypophysectomized rats were given plasma from normal human subjects (*vide* Results, Section 3). On the tenth day, animals were sacrificed. The adrenals were removed, dissected free of fat, wet-weighed, fixed in formalin, stained with Sudan Black, and sectioned for histologic study. Completeness of hypophysectomy was estimated by testicular size, and in six instances, by gross examination of the sella. All animals judged to be incompletely hypophysectomized were discarded.

RESULTS

1. *Bilateral adrenal hyperplasia*

Plasma for assay was obtained from nine patients with this disease. All showed the typical clinical and laboratory features of Cushing's syndrome. In six patients, the diagnosis of adrenal hyperplasia was proved at operation, and in the remaining three (N.Co., R.P., B.S.) no adrenal tumor was detected by presacral aerography. Eight of the nine patients (with the exception of S.A.) showed the typical plasma 17-hydroxycorticosteroid hyper-response to a standardized ACTH test (2, 9).

Table I shows results of the assay for adrenal weight-maintaining activity in the plasma of these nine patients. Eight of the nine plasma specimens (with the exception of patient B.S.) were found to prevent adrenal atrophy to a significant degree.

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TABLE I

Effect of plasma injection upon adrenal weight of hypophysectomized rats: Patients with Cushing's syndrome due to bilateral adrenal hyperplasia

Patient	Injected animals			Non-injected animals			Difference in paired adrenal weight	"t"*
	No. animals	Mean paired adrenal weight	S.D.	No. animals	Mean paired adrenal weight	S.D.		
		mg.			*mg.*		*mg.*	
F. S.	5	11.9	1.47	9	7.3	0.74	4.6	8.0
S. L.	6	10.7	1.27	9	7.3	0.74	3.4	6.4
N. Co.	6	9.8	1.34	6	7.6	0.84	2.2	3.4
C. P.	7	9.9	1.29	9	7.6	0.55	2.3	4.8
B. W.†	4	9.1	1.36	3	6.5	0.47	2.6	3.0
H. H.†	3	11.3	2.82	6	7.7	1.04	3.6	3.0
R. P.	8	11.0	1.09	9	8.0	0.41	3.0	7.6
S. A.	7	7.9	1.11	10	6.5	0.99	1.4	2.8
B. S.	6	8.9	1.73	6	7.8	0.30	1.1	1.5
Average		10.1			7.4		2.7	7.40‡

* Value of "t" here and in subsequent tables was computed by assuming equal variability in the injected and non-injected groups of animals (29).

† The small number of animals was the result of deaths during the injection period.

‡ The differences in mean paired adrenal weights yield a S.D. of 1.10 and consequently the S.E. of the average difference is 0.37 so that "t" is 7.40, significant at the 1 per cent level. If the differences in numbers of animals and the differences in variability in the injected versus the non-injected groups are taken into account by applying a weighting procedure, the value of "t" is nearly identical (7.2).

It should be pointed out that patients C.P. and H.H. had undergone bilateral subtotal adrenalectomy one and one-half and three years before the plasma was obtained for assay.

Since the numbers of animals were necessarily small (being limited by the amount of plasma available), data from individual experiments were pooled for additional statistical analysis. The average difference in adrenal weight between injected and non-injected animals was 2.7 mg. with a "t" value of 7.4 (Table I). The difference was significant at the 1 per cent level. It was of interest that injected animals in this and subsequent groups generally showed greater final body weights than uninjected animals, the difference averaging about 6 Gm. (In this group, average weight gain of injected animals was 9 Gm.; of uninjected controls, 4 Gm.) This was true regardless of whether the plasma contained any activity in maintaining adrenal weight or not. Calculation of the average correlation coefficient between body weight and adrenal weight gave an "r" value of 0.289, which is not significant (average slope, 0.04). The greater final body weight of injected animals could thus account for only about 0.2 mg. of the 2.7 mg. difference in adrenal weights.

It is recognized that the degree of adrenal weight maintenance is modest, with an average adrenal weight of 10.1 mg. in injected animals as compared with 7.4 mg. in the controls. However, when similarly hypophysectomized animals were injected with a standard ACTH preparation in massive doses, *i.e.*, 1 I.U. daily in three divided doses for nine days, adrenal weight averaged only 14.9 mg. Further, corticotropic material has been estimated to be present in amounts of the order of milliunits or fractions of milliunits per 100 ml. of human plasma (5, 14, 15), and each experimental animal in the present study received but 8 to 9 ml. plasma.

Histologic study of adrenal glands from animals injected with plasma revealed persistence of the sudanophobic zone, which was abolished by ACTH administration.

Studies performed in two patients (F.S. and H.H.) employing three animals in each assay failed to reveal adrenal ascorbic acid-depleting activity in plasma when tested by the Munson modification (16) of the Sayers technique (17).

2. *Adrenal cortical tumor*

The data obtained from the assay of plasma from three patients with documented adrenal cortical adenoma or carcinoma are shown in Table II. None of the three had previously shown excessive responses of plasma corticosteroid levels to the

TABLE II

Effect of plasma injection upon adrenal weight of hypophysectomized rats: Patients with Cushing's syndrome due to adrenal cortical tumor

Patient	Injected animals			Non-injected animals			Difference in paired adrenal weight	"t"*
	No. animals	Mean paired adrenal weight	S.D.	No. animals	Mean paired adrenal weight	S.D.		
		mg.			*mg.*		*mg.*	
V. C. (adenoma)	5	8.7†	1.08	10	9.1†	0.99	0.4	0.3
C. C. (carcinoma)	7	8.1	0.46	10	7.6	0.87	0.5	1.6
A. G. (carcinoma)	5	9.3	0.56	9	8.4	1.11	0.9	2.0

* P in each case > 0.05.

† The greater adrenal weights found in the study of plasma from patient V. C. may be related to the fact that this experiment was terminated after seven days of injection instead of nine days because of a large number of animal deaths. Post-hypophysectomy atrophy of the adrenal would not be expected to have reached its plateau within a seven day period.

standardized ACTH test (9), and no adrenal weight-maintaining activity could be demonstrated in their plasma.

3. *Normal subjects*

Table III shows results of the assay of plasma from normal individuals. The first specimen was taken from a single normal subject, while the remaining three specimens were pools of approximately equal plasma samples taken from three to five normal individuals. No significant increase of adrenal weight could be shown in the injected animals. Grouped data revealed an average difference in adrenal weight of injected versus uninjected animals of 0.05 mg. with a "t" value of 0.19 (not significant).

4. *Primary adrenal cortical insufficiency*

A summary of the results of assay of plasma from patients with adrenal insufficiency, either spontaneous or following bilateral total adrenalectomy, is shown in Table IV. Most assay methods have shown elevated levels of corticotropin in the blood of such patients (4–6, 18). It was therefore of interest to learn if plasma from adrenal-insufficient subjects also contained adrenal weight-maintaining activity. Blood was obtained and plasma pooled from five such patients whose exogenous steroid (cortisone) had been discontinued six to eight hours before blood was taken. Table IV shows that no adrenal weight-maintaining activity was detectable. This experiment does not offer conclusive evidence for the absence of adrenal

TABLE III

Effect of plasma injection upon adrenal weight of hypophysectomized rats: Normal subjects

Subject	Injected animals			Non-injected animals			Difference in paired adrenal weight	"t"
	No. animals	Mean paired adrenal weight	S.D.	No. animals	Mean paired adrenal weight	S.D.		
		mg.			*mg.*		*mg.*	
N. C.	6	8.0	0.64	6	7.6	0.84	0.4	0.9
Pool No. 1	9	7.8	1.12	5	7.6	0.32	0.2	0.4
Pool No. 2	7	9.4	1.19	10	9.1	0.98	0.3	0.6
Pool No. 3	6	7.7	0.99	9	8.4	1.11	0.7	1.2

Average difference in mean paired adrenal weights, injected versus control	0.05 mg.
S.E. of the difference (based on S.D.'s of individual experiments)	0.27
"t"	0.19
Degrees of freedom	50
p	>0.7

TABLE IV

Effect of plasma injection upon adrenal weight of hypophysectomized animals: Patients with adrenocortical insufficiency

	Injected animals			Non-injected animals				
	No. animals	Mean paired adrenal weight	S.D.	No. animals	Mean paired adrenal weight	S.D.	Difference in paired adrenal weight	"t"*
		mg.			mg.		mg.	
Plasma pool	6	8.1	0.52	5	7.6	0.33	0.5	1.8

* $p > 0.4$.

weight-maintaining activity from Addisonian blood since, as Bethune, Ganong, Hume, and Nelson have shown, the time elapsed after steroid withdrawal may not have been adequate to permit "rebound" of anterior pituitary function from the suppressive effect of the steroid (18). However, studies with an aliquot of this pooled plasma revealed a decrease of 43 per cent in the adrenal ascorbic acid of two animals as compared with two controls (16).

5. *Pregnancy*

Since women in the third trimester of normal pregnancy had been previously shown to exhibit exaggerated plasma 17-hydroxycorticosteroid response to ACTH [4] like that found in Cushing's syndrome (2), a study of adrenal weight-maintaining activity in the plasma of this group was also made. Table V shows results of assay of plasma pooled from three groups of pregnant women, each group comprising two to three women. All three pools showed significant activity.

6. *Acromegaly*

Because several workers (10–12) had observed adrenal weight-maintaining properties in pituitary growth hormone fractions, studies of plasma were

[4] There is evidence that this "excessive response" may be in part related to a delayed disposal of hydrocortisone from plasma of pregnant women (19, 20).

TABLE V

Effect of plasma injection upon adrenal weight of hypophysectomized animals: Women in the third trimester of normal pregnancy

	Injected animals			Non-injected animals				
	No. animals	Mean paired adrenal weight	S.D.	No. animals	Mean paired adrenal weight	S.D.	Difference in paired adrenal weight	"t"*
		mg.			mg.		mg.	
Pool No. 1	5	10.4	1.77	10	7.6	0.87	2.8	3.0
Pool No. 2	8	9.7	1.16	10	6.5	0.99	3.2	5.8
Pool No. 3	6	11.0	1.67	6	7.8	0.28	3.2	4.2

* p in each case < 0.01.

TABLE VI

Effect of plasma injection upon adrenal weight of hypophysectomized rats: Two patients with active acromegaly

	Injected animals			Non-injected animals				
Patient	No. animals	Mean paired adrenal weight	S.D.	No. animals	Mean paired adrenal weight	S.D.	Difference in paired adrenal weight	"t"*
		mg.			mg.		mg.	
F. N.	6	9.9	1.13	9	7.6	0.55	2.3	4.7
G. E.	8	10.9	0.89	9	8.0	0.41	2.9	8.3

* p in both instances < 0.01.

TABLE VII

Effect of growth hormone upon adrenal weight of hypophysectomized rats

Injected animals			Non-injected animals				
No. animals	Mean paired adrenal weight	S.D.	No. animals	Mean paired adrenal weight	S.D.	Difference in paired adrenal weight	"t"*
	mg.			mg.		mg.	
5	10.8	0.53	7	8.8	1.09	2.0	4.2

* $p < 0.01$.

also made in two patients with "active" acromegaly. Table VI shows significant adrenal weight-maintaining activity in the plasma of both patients. It should be pointed out that neither of these two subjects had exaggerated plasma corticosteroid responses to ACTH.

7. Growth hormone

Investigation of the effect of bovine growth hormone (supplied by the National Institutes of Health) was carried out. Three mg. of this preparation were administered daily to a group of hypophysectomized rats, the adrenal weight assay being carried out as described above. The data presented in Table VII indicate that this growth hormone preparation contained significant adrenal weight-maintaining activity ($p < 0.01$) as previously demonstrated (10–12). The sudanophobic zone of the adrenal cortex persisted. One mg. of this somatotropin contained no corticotropic activity, as determined by the method of Saffran and Schally (21).

DISCUSSION

The data presented indicate that plasma from patients with Cushing's syndrome due to bilateral adrenal hyperplasia exhibited adrenal weight-maintaining activity as demonstrated by assay in hypophysectomized rats. Plasma of pregnant women in the third trimester of pregnancy and of two patients with active acromegaly showed similar activity. It is not possible at this time to ascribe this weight-maintaining effect to a hormone with any certainty, since it is recognized that the demonstration of a substance of possibly hormonal nature conventionally rests upon the finding of its differential potency at increasing dose levels. Attempts to assay patients' plasma at doses greater than 0.9 ml. per rat per day in this study proved impractical since the larger amounts resulted in the death of nearly all the test animals. Construction of a dose-response curve must await purification of plasma.

A number of substances might be implicated to explain the partial maintenance of adrenal weight observed in these studies: 1) steroids of the adrenal cortex, 2) corticotropin, and 3) growth hormone. Certain steroids have been shown to cause increases in adrenal weight (22, 23), but in the present study it seems unlikely that elevated plasma adrenal steroid levels were responsible for the observed increase in adrenal weight. In the first place, no adrenal weight increase was induced by the plasma from patients with adrenal cortical tumors whose resting plasma corticosteroid levels were as high as or higher than those of patients with adrenal hyperplasia.[5] Second, adrenal weight-maintaining activity was found in plasma of two patients who had undergone bilateral adrenalectomy before the assay was carried out. Third, cortisone (a prototype of the class of steroid elevated to the greatest degree in Cushing's syndrome) has been shown to have no weight-augmenting effect upon adrenals of hypophysectomized rats (23).

Corticotropin has also been suspected as a possible etiologic factor in bilateral adrenal hyperplasia. Although some workers have claimed to find corticotropin in the plasma of such patients (24, 25) most observers have failed to detect increased amounts of this substance by a variety of assay procedures (4–7). Furthermore, administration of the usual commercial preparations of ACTH has not been shown to cause the degree of adrenal cortical hyper-response found in patients with adrenal hyperplasia (8, 9).

[5] Average plasma 17-OH-corticosteroid levels in 15 patients with bilateral adrenal hyperplasia, 29 micrograms per 100 ml.; average level in six patients with adrenal cortical tumor, 43 micrograms per 100 ml. (9).

Since it seems unlikely that either adrenal steroids or ACTH can account for the observed adrenal weight-maintaining activity, the question of a possible role of other hormones may be raised. Liddle, Island, Rinfret, and Forsham (26) studied a material prepared by Rinfret from equine pituitaries which exhibited adrenal weight-maintaining activity and was capable of enhancing the sensitivity of the human adrenal cortex to "orthodox" ACTH, as measured by excretion of urinary corticosteroids. This equine pituitary extract in itself had very little steroidogenic effect. It is not known whether or not the weight-maintaining "substance" in plasma of patients with adrenal hyperplasia has the property of enhancing the steroidogenic effect of ordinary ACTH upon the adrenal cortex.

Adrenal weight-maintaining effects have been found in growth hormone fractions by Reinhardt, Geschwind, and Li (10), Young and his associates (11), and Cater and Stack-Dunne (12). In the study reported here, growth hormone showed partial adrenal weight-maintaining activity as did the plasma of two patients with acromegaly in whose blood one might expect to find excessive somatotropin (27). It will be recalled that these two acromegalic patients did not show adrenal cortical hyper-response to ACTH.

The finding of adrenal weight-maintaining activity in the plasma of pregnant women in the third trimester is of interest in view of the alleged hyperresponsiveness of the adrenal cortex (2). The relationship, if any, between the two observations is not clear. It may perhaps be relevant that increased growth hormone levels have been found by Contopoulos and Simpson (28) in blood of pregnant rats, and that Cater and Stack-Dunne (12) have detected adrenal weight-maintaining activity in human placental extracts. No claim can be made on the basis of the data presented that either the adrenal hypersensitivity or the adrenal weight-maintaining activity in human pregnancy blood is or is not related to growth hormone. Furthermore, with our limited knowledge, it is impossible to decide at this time whether the adrenal weight-maintaining activity found in Cushing's syndrome with adrenal hyperplasia, acromegaly, and pregnancy is due to the same substance or to different substances.

SUMMARY

1. Adrenal weight-maintaining activity was detected in plasma of patients with Cushing's syndrome due to bilateral adrenal hyperplasia. The assay used was the prevention of complete adrenal atrophy in hypophysectomized rats. No adrenal weight-maintaining activity was found in plasma of three patients with Cushing's syndrome due to adrenal tumor, in normal subjects, or in a pool of plasma from patients with primary adrenal cortical insufficiency.

2. Adrenal weight-maintaining activity was observed in three pools of plasma from women in the third trimester of pregnancy and in two patients with active acromegaly.

3. On the basis of this study, no statement can be made concerning the nature or origin of the substance or substances responsible for adrenal weight-maintaining activity in bilateral adrenal hyperplasia, late pregnancy, or active acromegaly.

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OBSERVATIONS ON THE MINOR BASIC HEMOGLOBIN COMPONENT IN THE BLOOD OF NORMAL INDIVIDUALS AND PATIENTS WITH THALASSEMIA

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In a previous report (1) a second hemoglobin was described which was found at a concentration of approximately 3 per cent in the blood of normal adult individuals. It was found to resemble hemoglobin E in electrophoretic properties, but identity with this abnormal hemoglobin was not established. This same component probably had been observed by previous investigators employing the classical Tiselius procedure in occasional specimens of blood from normal persons (2, 3) and thalassemia patients (4), but had not been defined as a hemoglobin. The present report describes further properties of this hemoglobin (designated A_2 in this paper) with particular reference to its constant presence in the blood of humans and certain primates and its elevation in individuals with thalassemia trait.

MATERIALS AND METHODS

Blood specimens were usually obtained in acid citrate dextrose or Alsever's solution. Hemolysates were prepared by the method of Drabkin without the use of $AlCl_3$ (1). Following hemolysis the material was subjected twice to high speed centrifugation to insure removal of all red cell ghosts. Special care was necessary in the preparation of the hemoglobin solutions because even a small number of ghosts in clear-appearing solutions interfered in the procedures of electrophoresis employed. The ghosts became adsorbed at the origin, trapping hemoglobin, which then gradually became released during the experiment causing a trail of hemoglobin extending from the origin. Fresh blood samples were routinely converted to carboxyhemoglobin both before and again after the preparation of the hemolysates. This was done by swirling the red cells or hemoglobin solutions under a jet of CO in a large tube for approximately four minutes. The cyanmethemoglobin derivative was used primarily for older specimens of blood or hemoglobin where partial conversion to methemoglobin had occurred. To each 1-ml. sample 0.05 ml. of freshly prepared 1 M potassium ferricyanide and 0.05 ml. of 1 M potassium cyanide were added.

Normal blood was obtained chiefly from laboratory workers. Material from patients with various diseases was collected from a number of New York hospitals. The specimens from patients with thalassemia were obtained from Italian and Greek individuals in the hematology clinic at the Babies Hospital, Columbia-Presbyterian Medical Center, New York. Five individuals with thalassemia in two families were obtained from index cases at Bellevue Hospital. The great majority of thalassemia trait cases were parents of children with severe Cooley's anemia. In all instances the diagnosis of thalassemia was based on the hematological picture. Several individuals who were suspected of having thalassemia were excluded from the study because of uncertain hematological findings.

Zone electrophoresis was carried out by a slight modification of procedures described previously (5). A broad starch slab, thinner than those usually used, was found most suitable. The following dimensions were employed: 32 by 45 by 0.7 cm. The starch was washed over a filter (with buffer only) prior to use. Barbital buffer pH 8.6 $\Gamma/2$ 0.05 was routinely employed. The hemoglobin solution was applied to the slab by means of a capillary tube which was inserted in a slit 1 to 2 cm. in length in the starch which was kept just dry enough to prevent fusion of the slit. Numerous specimens could be applied to a single slab in this manner (as many as 30 in three rows in some experiments). Following application of one row of specimens, the slits were fused by means of buffer pipetted carefully on the starch several cm. on either side of the row. A small spatula was used to flatten the site of application. The proper application of the hemoglobin in a thin band required considerable attention to details, but with some experience it was readily possible.

For the demonstration and quantitation of the A_2 component, the concentrated hemoglobin solutions obtained by the Drabkin technique were used. For the quantitation and clear delineation of the abnormal hemoglobins, the concentrated solutions were diluted with two volumes of buffer. Three-tenths ml. of either the concentrated or dilute solution was usually applied. The starch slab was covered with a polyethylene sheet which was applied with considerable care after blotting the block to remove excess moisture. Too moist a block was avoided because of "melting" of the slab under the pressure of application of the cover. The block was kept so that it would just adhere to the polyethylene under gentle pressure. The sheet was applied in such a manner as to force out most of the air between it and the starch. The

separation was carried out for approximately 16 hours at 380 volts, giving a current without heating in the vicinity of 85 ma. Following the separation, a dry towel was placed at both ends of the block after removal of the cloth contacts to the electrode vessels. This blotting procedure removed excess moisture that accumulated during the separation and facilitated removal of the polyethylene sheet and other manipulations of the starch block. The slab was then carefully viewed over a strong light which caused the red hemoglobin spots to stand out very clearly. In work with the quantitation of the A_2, this spot was outlined with a thin spatula and the division from the main A component marked. The two segments which included these components were partitioned and removed from the lower polyethylene sheet, and each was packed on a ground glass filter. Distilled water was then added to displace the hemoglobin from the starch. A volume of water slightly greater than the volume of the packed starch was usually sufficient to remove all hemoglobin. With some preparations of starch a few of the smaller particles passed through the filter. These were removed by centrifugation. The main A fraction was diluted to 20 times the volume of the A_2 fraction and each was measured in a Beckman spectrophotometer at 540 mμ. The per cent concentration of the A_2 component was obtained from these readings employing the dilution factor of 20. The protein was also determined on the same fractions by means of the modified Folin procedure (5). Hemoglobin A was used as the standard for these measurements of color and protein concentration. The carboxyhemoglobin color procedure was routinely used but the cyanmethemoglobin method was very helpful in many instances. The two color procedures gave very similar results. When the carbonmonoxy method was used, considerable care was necessary to keep the hemoglobin in the CO form. Partial formation of methemoglobin usually was greater for the A_2 component and gave low values. Direct protein analysis gave values for the A_2 component which were 5 to 10 per cent higher than by the hemoglobin color procedures.

TABLE I

Repeated analysis of same hemoglobin solution (normal)

Hemoglobin A_2 expressed as per cent of total hemoglobin

	Same starch blocks	Different starch blocks
	2.41	2.51
	2.51	2.33
	2.18	2.45
	2.11	2.98
	2.36	1.92
	2.93	2.51
	2.42	2.75
	2.65	2.62
Mean	2.56	2.51
S.E.	±0.09	±0.11
Coefficient of var.	9.9%	12.4%

This difference was found to be due primarily to contamination of these preparations with non-colored proteins.

One experiment with a normal hemoglobin solution separated eight times as carboxyhemoglobin on the same starch block is shown in Table I. The results for the same material separated eight times on different starch blocks are also illustrated. In these experiments the results are quite similar. However, there was no question about the greater reproducibility using the same starch slab, and for accurate comparisons this procedure was usually employed.

Antisera for immunological analyses were produced by injecting whole hemoglobin solutions from normal individuals. The Freund adjuvant technique was employed in eight rabbits. Three of these showed weak antibodies against hemoglobin three months after injection. These were re-stimulated by the Freund technique and potent antisera were obtained four weeks after the second injection.

Fetal hemoglobin was measured by the procedure of Singer, Chernoff, and Singer (6, 7). Solubility studies were carried out by the procedure described by Green, Cohn, and Blanchard (8).

RESULTS

I. *Separation of hemoglobin A_2 from solutions of normal hemoglobin*

Figure 1 illustrates the protein curve obtained after separation by zone electrophoresis of the hemolysate from the red cells of a normal individual. Two colored components are visible—the main hemoglobin A and the small hemoglobin A_2. The latter is the subject of this report. In addition, a third small peak is observed close to the site of application. This is a colorless component which showed some variation in concentration in different blood specimens and is the main non-hemoglobin protein constituent of the red cells.[1] The experiment illustrated in Figure 1 was carried out with carboxyhemoglobin and the color of the A_2 component was clearly visible against the white starch background at the site of the A_2 protein peak. Figure 2 shows the carboxyhemoglobin color of the hemolysates from the blood of nine normal individuals and two with sickle-cell trait. The A_2 component is visible in all these specimens. It was also observed in a similar position relative to the main A hemoglobin when oxyhemoglobin,

[1] The slowly migrating colorless protein is often elevated in thalassemia and may be confused with hemoglobin A_2 when protein analyses not involving hemoglobin color are carried out by procedures such as staining of filter paper strips and free solution electrophoresis.

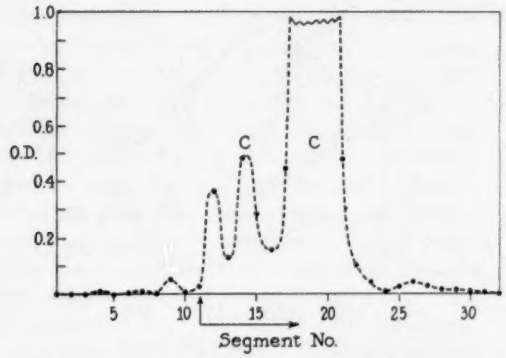

FIG. 1. PROTEIN CURVE OBTAINED AFTER ELECTROPHORETIC SEPARATION OF THE HEMOLYSATE FROM THE RED BLOOD CELLS OF A NORMAL INDIVIDUAL

The two peaks labeled C were colored. The arrows represent the origin and the migration to the anode.

methemoglobin, and cyanmethemoglobin was used. The color, characteristic of these derivatives, was visible for both the A component as well as the A_2. The two brown methemoglobin spots were both slightly slower than the corresponding carbonmonoxy derivatives; the port wine colored cyanmethemoglobin spots on the other hand were slightly faster. The A_2 component was clearly not a modified form of hemoglobin A due to a different hemoglobin derivative.

In order to rule out the possibility of the anticoagulant playing a role in these studies, preparations of hemoglobin were obtained with heparin, oxalate, citrate and clotted blood. All preparations showed the A_2 component in approximately the same amount. In addition, different procedures for laking the red cells were employed. The use of water alone without toluene and also simple repeated freezing and thawing followed by centrifugation furnished hemoglobin solutions that all showed the same A_2 component. In this work the importance of complete removal of the red-cell ghosts prior to separation in the starch became apparent. This was best accomplished through the use of toluene and repeated high speed centrifugation. A red streak extending from the site of application on the starch which obscured the A_2 component was observed if all cell ghosts were not eliminated.

Separation by zone electrophoresis was also carried out in a polyvinyl chloride or a paper pulp supporting medium, and the A_2 component was clearly apparent. It could also be observed with some difficulty on a filter paper strip particularly after staining the paper. However, the small amount of material that can be applied to the paper strip and the slight trailing of hemoglobin A hampered clear recognition of hemoglobin A_2 by this procedure.

Numerous experiments were carried out with hemoglobin A which had been isolated free of A_2. After salt precipitation, exposure to acid and alkaline pH, treatment with urea and prolonged dialysis and concentration, it was examined by zone electrophoresis. An increase in fast migrating material which formed a streak from the main A peak was frequently observed. However, in no instance was any material formed which resembled the A_2 component.

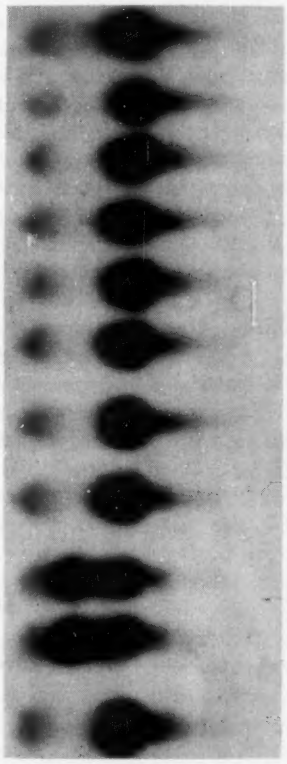

FIG. 2. PHOTOGRAPH OF A BROAD STARCH BLOCK SHOWING THE RED HEMOGLOBIN COMPONENTS SEPARATED FROM THE HEMOLYSATES PREPARED FROM THE RED CELLS OF EIGHT NORMAL INDIVIDUALS AND TWO WITH SICKLE-CELL TRAIT

II. *Properties of the isolated A_2 hemoglobin*

The A_2 component could be readily isolated free from all hemoglobin A by preparative zone electrophoresis. Figure 3 shows the electrophoretic curve obtained for one such preparation after concentration by ultrafiltration. The curve for hemoglobin color closely parallels that for protein as determined by the modified Folin reagent. No peak is visible at the point where hemoglobin A should appear as indicated from a whole hemoglobin preparation separated on the same starch block. These concentrated preparations of hemoglobin A_2 also showed from 5 to 10 per cent more protein when compared with hemoglobin A in terms of hemoglobin color.

Examination of this material in the ultracentrifuge showed essentially one component, although evidence of small amounts of contaminating proteins was obtained following prolonged centrifugation. The A_2 preparation was compared directly with hemoglobin A in the same experiment and at the same concentration. No difference in sedimentation rate was detectable between the major A_2 component and hemoglobin A.

Figure 4 shows the absorption curves for the isolated A_2 hemoglobin. These are indistinguishable from those obtained for hemoglobin A and indicate that the A_2 component is a true hemoglobin.

Some difficulties were encountered in working with the A_2 component because of ease of denaturation. If the material were separated in the CO form and isolated and concentrated in the cold under optimal conditions, it retained the discrete

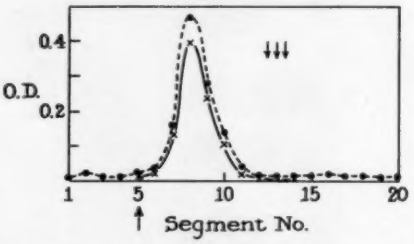

FIG. 3. PROTEIN CURVE AFTER ELECTROPHORETIC SEPARATION OF ISOLATED A_2 COMPONENT

The three arrows indicate the position A hemoglobin would occupy if present. The dotted line represents Folin color; the solid line carboxyhemoglobin color.

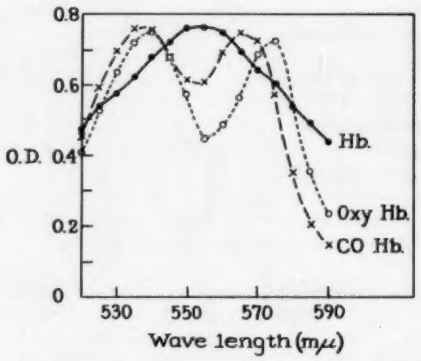

FIG. 4. ABSORPTION CURVES OBTAINED FOR VARIOUS DERIVATIVES OF A_2 HEMOGLOBIN

mobility found in the whole hemoglobin solution. However, on standing even for short periods at 4°C. alterations were observed frequently and a broader peak was obtained on electrophoresis with slight shifts in mobility. In addition, a large mobility change was occasionally seen for a portion of the A_2 material; it would show a much slower mobility. This change with resulting double bands was also observed in rare instances when whole hemolysates were separated. These were usually from blood which had been shipped considerable distances. The question of whether hemoglobin A_2 represented a single species of hemoglobin was never entirely answered. In some instances the colored bands and the peaks derived from them were broader than might be expected from a single component. However, efforts attempting further separation under varying conditions of pH and ionic strength in the granular starch medium were unsuccessful. When clear splitting of the A_2 band was obtained, it was always due to partial conversion to methemoglobin or some other secondary product. Separation in a polyvinyl chloride medium and also electrophoresis in starch gels [2] have indicated the presence of a faint hemoglobin band which migrates slightly slower than the main A_2 component. This has not been isolated and the question of whether or not it represents a derivative of A_2 hemoglobin remains unanswered.

These alterations and the relatively small amount of material available limited such studies as the determination of solubility. When good

[2] Carried out by Dr. A. G. Bearn.

preparations were employed, the solubility in the ferrohemoglobin form at different ionic strengths of phosphate buffer (pH 6.8) was close to that for hemoglobin A. The curve obtained for A_2 hemoglobin when these results were plotted in the standard manner was just below that for hemoglobin A, suggesting a slightly lower solubility.

Considerable effort was directed toward the crystallization of the A_2 component. When hemoglobin was crystallized by the method of Drabkin from crude hemolysates (one experiment), the A_2 component was found at the same concentration as in the original solution. A crystalline preparation obtained through the courtesy of Dr. Drabkin also showed the A_2 component at a concentration of 2.5 per cent. These results suggested that A_2 hemoglobin crystallized in a similar manner to A hemoglobin. However, the difficulty in detecting other crystal forms at the low concentration of the A_2 fraction requires consideration. Attempts were made to crystallize the A_2 component from fresh preparations isolated by zone electrophoresis. Some crystals were obtained but the double pyramid type characteristic of A hemoglobin was not observed. For carboxyhemoglobin, fan-shaped, rounded forms were found; whereas for methemoglobin, long thin needles, which frequently grouped into fan-like aggregates, were found. Hemoglobin A isolated by electrophoresis formed the double pyramids just as in the case of the whole hemoglobin solution. The exact reason for these differences could not be determined. The difficulties involved in the isolation and preservation of A_2 hemoglobin must be considered in the interpretation of these results.

An attempt was made to determine if A_2 hemoglobin was immunologically different from hemoglobin A. Two different potent antisera against unfractionated hemoglobin reacted strongly with highly purified preparations of both A and A_2 hemoglobins. Quantitative precipitin curves with the two preparations were very similar. Absorption of one of these antisera with either A hemoglobin or A_2 hemoglobin removed the antibodies against the other. Analysis by the Ouchterlony plate method showed fusion of the major band for A and A_2 hemoglobins. However, secondary lines appeared with both preparations of hemoglobin, indicating that they were not immunologically homogeneous. All these experiments pointed to a close immunological relationship between the two hemoglobins.

III. *Distribution of A_2 hemoglobin in different red cells*

An attempt was made to determine whether A_2 hemoglobin was present at a constant percentage in all red blood cells or whether it represented the predominant hemoglobin of certain unusual forms. In view of the fact that certain red blood cells show increased osmotic resistance, particularly in individuals with thalassemia where the A_2 component is elevated, the possibility arose that the cells resisting hemolysis at low-salt concentrations might be high in A_2 hemoglobin. Two experiments were carried out, one with cells from a normal individual, the other with cells from a patient with thalassemia trait. In each instance, more than 90 per cent of the cells were hemolyzed. The remaining more resistant cells were then washed with saline and hemolyzed by the usual procedure. Quantitation of the A_2 component following electrophoresis indicated a concentration of 2.3 per cent compared to 2.9 per cent for the original whole normal blood, and 5.2 per cent compared to 5.1 per cent for the thalassemia blood. The more resistant cells did not show a higher concentration of the A_2 component.

Studies were also performed to determine whether the specific activity of the iron in hemoglobin A_2 was the same as that in the main A component at various times following administration of Fe^{59}. Analyses were carried out on the fifth day and on the twelfth day following the administration of Fe^{59} to a normal individual. The A and A_2 components were isolated free of each other and the radioactivity determined both in terms of Fe concentration and cyanmethemoglobin color. By each procedure the specific activity was similar for the two components, although in both instances the specific activity was slightly lower for the A_2 hemoglobin. During the course of these experiments, it was observed that the faster migrating hemoglobin at the front edge of the A peak showed a considerably lower specific activity than the major slower fraction. Since these fractions overlapped it was difficult to determine the exact

specific activity of A hemoglobin for comparison with the A_2 fraction. Further studies are continuing and will be published in detail separately (9).

IV. *A_2 concentration in normal individuals and various hematological disorders*

Fresh hemoglobin preparations from two individuals, one with a normal and the other with an elevated level of A_2 hemoglobin, were examined on seven occasions over a period of four months' time. The A_2 component was quantitated in terms of carboxyhemoglobin color, and the values obtained are shown in Table II. This experiment demonstrated that the level of the A_2 component was quite constant in the same individual and the variation observed was within the experimental error of the method of analysis.

Tables III and IV summarize the results obtained for the quantitation of the A_2 component in 155 different individuals. In each case it was determined by measuring the carboxyhemoglobin color. A mean value of 2.54 per cent was found for normal individuals in this series of cases. Many other normal blood specimens were analyzed but they are not included because they were not as strictly comparable to the pathological specimens. The methods employed varied for these other specimens and cyanmethemoglobin color was often measured. However, in no proven instance was a level below 1.8 per cent or higher than 3.2 per cent obtained for normal individuals in either group. Lower values were occasionally found but these were due to selective partial conversion of the A_2 component to methemoglobin. When these were checked by the cyanmethemoglobin method, these low values were proved erroneous. No significant difference could be found in the A_2 concentration for white and Negro individuals. Specimens from five persons of Chinese descent also showed similar A_2 levels.

TABLE II

Repeated analysis of hemoglobin A_2 from different bleedings of the same two individuals over a six-month period

	Normal	Carrier of thalassemia trait
	2.41	4.51
	2.35	4.80
	2.42	6.03
	2.18	5.53
	2.13	4.61
	2.35	5.12
	2.36	6.52
	2.55	4.82
	2.61	4.66
	2.42	4.54
	3.11	5.51
	1.93	5.81
Mean	2.40	5.22
S.E.	±0.08	±0.19
Coefficient of var.	11.5%	12.6%

Blood specimens were obtained from 44 patients suffering from various diseases other than thalassemia. Approximately one-half of these came from individuals with assorted blood disorders (Table III). In no instance was an elevation of the A_2 component encountered. The mean value of 2.19 with a standard deviation of ± 0.62 per cent (Table IV) was slightly lower than for the normal group ($p \sim 0.01$). However, because this represented a very heterogeneous series, a considerably larger number should be studied for a proper comparison. Two low samples, one from a patient with atypical sprue (1 per cent) and the other from a case of idiopathic hemolytic anemia (1.3 per cent), were re-analyzed in triplicate and were clearly lower than normal. Specimens from five patients with iron deficiency anemia were studied. All values were either low or in the normal range.

Thirty-four different individuals with thalassemia trait were studied in regard to the relative concentration of the A_2 component. Presumptive evidence for this diagnosis was obtained on the basis of a suggestive hematological picture existing in what were considered to be thalassemia families or as parents of children with Cooley's anemia. A mean value of 5.11 ± 1.36 was obtained (Table IV). Only two cases in the entire thalassemia series were in the normal range. Both of these were mothers of patients with Cooley's anemia and their hematological picture was compatible with thalassemia trait. Second blood specimens checked the initial findings in both instances. Levels of 2.4 and 2.2 per cent were obtained. One of these individuals was unusual in that she had 14 per cent fetal hemoglobin; in the other case there was 4 per cent fetal hemoglobin. One other individual in a Greek family with thalassemia showed relatively low levels with a mean value at 3.5 per cent (five

TABLE III

Per cent hemoglobin A_2 observed in normal and pathological conditions and the number of cases at each A_2 level

A_2	Normals	Thalass. trait	Thalass. major	Other diseases*	A_2	Normals	Thalass. trait	Thalass. major	Other diseases*
%					%				
1.0				1 w	3.5		1	1	
1.1				2 kn	3.8		1		
1.3				2 df	4.1		1	1	
1.4			1	1 n	4.2		2		
1.5				3 fko	4.3		1		
1.6			1	3 kiv	4.5		2		
1.7				1 l	4.6		1		
1.8	3		1	4 dkjr	4.7		3		
1.9	1		1		4.9		1		
2.0	3			2 gr	5.0		1		
2.1	4	1	1		5.1		2		
2.2			1	2 ho	5.2		2		
2.3	6	1		2 cr	5.3		2		
2.4	7		1	1 u	5.5		1		
2.5	6			1 n	5.6		2		
2.6	9			4 ekst	5.7		1		
2.7	6		1	5 jjjrr	5.9		2		
2.8	8		1	2 bs	6.1		2		
2.9	6			1 q	6.2		1		
3.0	3			3 imt	6.4		1		
3.1	2			4 ajps	8.1		1†		
3.2	1				9.5		1†		
3.4			1						
Totals examined:						65	34	12	44

* Other diseases as follows (in brackets are number of cases observed):

a—Aplastic anemia [1]
b—Extra-medullary hemopoiesis [1]
c—Hemochromatosis [1]
d—Hemolytic anemia [2]
e—Hemophilia [1]
f—Hypoproteinemia [2]
g—Hodgkin's [1]
h—Idiopathic thrombopenic purpura [1]
i—Leukemia [2]
j—Liver cirrhosis [5]
k—Iron deficiency [5]
l—Myeloma [1]
m—Nephritis [1]
n—Neoplasia [3]
o—Lead poisoning [2]
p—Peptic ulcer [1]
q—Pneumonia [1]
r—Secondary anemia [5]
s—Sickle-cell anemia [3]
t—Sickle-cell trait [2]
u—Uremia [1]
v—Tuberculosis [1]
w—Sprue [1]

† Thalassemia intermediate.

analyses). The remainder of the cases showed values that were clearly distinguishable from the normal. The highest level was 9.5 per cent (mean of four determinations) in one patient. This 25 year old patient was of interest because he consistently showed extremely high levels of fetal hemoglobin—values ranging from 25 to 55 per cent were obtained. However, he had not required transfusions. Another adult patient with 23 per cent fetal hemoglobin showed 8.1 per cent of the A_2 component. Both of these cases with the highest levels had been classified clinically as

TABLE IV

A_2 levels in different groups of individuals

Group	No. examined	% A_2 (Mean)	Standard error	Standard deviation	Coeff. of var.
					%
Normals	65	2.54	0.04	0.35	13.8
Carriers of thalassemia trait	34	5.11	0.23	1.35	26.5
Thalassemia major	12	2.51	0.25	0.85	33.8
Other miscellaneous diseases	43	2.19	0.10	0.62	28.3

TABLE V

t tests on A_2 levels in different groups

Groups compared	d.f.	t	p
Normals vs. thal. trait	97	14.43	<0.001
Normals vs. thal. major	75	0.64	>0.20
Thal. trait vs. thal. major	44	7.12	<0.001

unusual forms of thalassemia possibly of the intermediate type.[3]

Blood specimens from 12 patients with Cooley's anemia were examined (Tables III and IV). The mean level, 2.51, was close to the normal but the variation was considerable with some patients showing elevated levels and others depressed levels.[4] Five individuals with the double heterozygous condition, sickle-thalassemia, were also investigated. Exact quantitation of the A_2 component was difficult in the presence of sickle-hemoglobin. The impression was gained that the A_2 component was slightly elevated in concentration. It appeared to be normal in concentration in patients with sickle-cell trait (Figure 2) and sickle-cell anemia. However, five specimens of human blood were obtained from the umbilical cord of newborn infants. In each case the A_2 component was either absent or greatly diminished. One eight day old child who had 81 per cent fetal hemoglobin showed no evidence of the A_2 component.

V. *Statistical analyses*

The distribution of the A_2 values among healthy individuals approach normality but for a slight excess of low values. The distribution among carriers of thalassemia trait is also compatible with normality, except for four values mentioned above (2.1, 2.3, 8.1, 9.5), which have only a probability of about 1 per cent of belonging to the same population. These four values are responsible for the higher variance of the thalassemia group in comparison with normals (Table IV). The tests of significance (Table V) show that the difference of A_2 levels between normals and thalassemia carriers is highly significant. Beherens' d tests (10), when the variances of the two groups are significantly different, have given the same results as the t tests. The best discriminating point x_E between these two groups can be calculated according to the procedure of Cavalli (11) using the formula

$$x_E = \frac{x_1\sigma_2 + x_2\sigma_1}{\sigma_1 + \sigma_2},$$

where x_1 and σ_1 are the mean and the standard deviation for the thalassemia group; x_2 and σ_2 are the mean and the standard deviation for the normals: in our case x_E equals 3.09. The formula

$$t = \frac{x_2 - x_1}{\sigma_1 + \sigma_2} = 1.5$$

permits calculation of the error of classification from the table of probits. For t equals 1.5, a probability of 94 per cent is found (corresponding to 5 plus t equals 6.5). In other words, according to the data herein presented, on the basis of the A_2 levels only, all individuals with more than 3.1 per cent of A_2 can be classified as thalassemia trait. Such a classification has a probability of being correct of approximately 94 per cent.

VI. *Observations in other primates and certain animal species*

Five different types of monkeys were investigated in relation to the presence of the A_2 component. Blood was obtained from 10 chimpanzees and all specimens showed the A_2 hemoglobin. The picture obtained for these animals was indistinguishable from the human; the mobility of the A and the A_2 hemoglobin was very similar in the two species (Figure 5). Quantitation of the A_2 hemoglobin in two of these animals showed a concentration of 2.3 and 2.5 per cent. The South American monkey, *Cebus capucinus,* also showed the A_2 hemoglobin. However, in this case, levels from 4 to 5.5 per cent were obtained in the three animals examined. Numerous *Macacus rhesus* and *cynomolgous* monkeys were also studied. In no instance was any A_2 hemoglobin detectable. The West African green monkey, *cercopithecus,* also failed to show the A_2 component in the two blood specimens studied.

No abnormal hemoglobins were encountered in

[3] Blood from a third patient with an intermediate type thalassemia, kindly furnished by Doctor Hermann Lehmann, showed 10.8 per cent A_2 hemoglobin.

[4] Most of these patients had received transfusions one to six weeks prior to testing, and although fetal hemoglobin was high in all cases, some influence of the transfusions on the A_2 level appears probable.

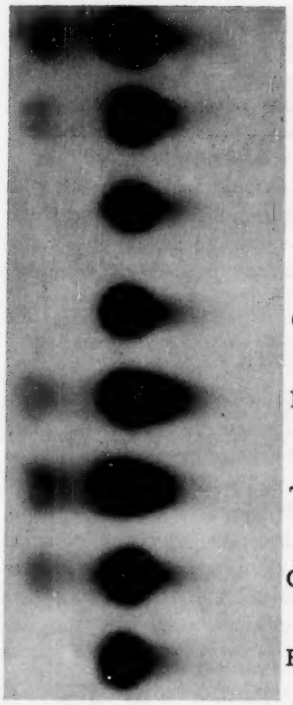

FIG. 5. COMPARISON OF THE HEMOGLOBIN PATTERNS OBTAINED FOR THE HEMOLYSATES OF HUMANS AND CERTAIN OTHER PRIMATES

The absence of an A_2 component in the blood of *rhesus* and *cercopithecus* monkeys is noted.

these monkeys except in the case of the *cynomolgous* type. Here all animals showed a fast hemoglobin in addition to the usual A hemoglobin. The relative amount of the fast type ranged from 25 to 65 per cent in the six animals studied. Examination of blood specimens from cattle, horses, pigs, dogs and rabbits failed to show a minor hemoglobin component similar to A_2 hemoglobin.

DISCUSSION

The properties observed for the A_2 component in the present study indicate that this hemoglobin is closely related to the main hemoglobin A. The only clearly defined difference is in electrophoretic mobility. Immunologically, the two hemoglobins could not be distinguished. Hemoglobin A_2, thus, is not another hemoglobin of the type represented by fetal hemoglobin but appears to resemble the abnormal hemoglobins which also show a close relationship to normal hemoglobin A. It resembles the abnormal hemoglobin E most closely, but absolute establishment of identity has not been possible. The virtual absence of A_2 hemoglobin in the blood of newborn infants and its appearance after birth with the decline in fetal hemoglobin is analogous to the situation which has recently been described for hemoglobins S, C and E (12–14).

The significance of the presence of hemoglobin A_2 in the blood of all normal adult individuals is not apparent. The appearance of the abnormal hemoglobins, which are considered by many workers (15–17) to represent allelic forms of hemoglobin A, does not seem to affect the concentration of the A_2 component; normal levels were found both in the heterozygous and homozygous types of sickle-cell disease. This suggests that A_2 hemoglobin synthesis is not affected by different mutants of the hemoglobin locus. The occurrence of a hemoglobin similar to the A_2 component in the blood of certain other primates, particularly the chimpanzee, is of some anthropological interest. The possibility is raised that A_2 hemoglobin may represent some type of vestigial protein.

Elevation of the A_2 component is a characteristic finding among individuals with the thalassemia trait. However, low levels were usually found in patients with Cooley's anemia. This is rather surprising when one considers that Cooley's anemia corresponds to homozygosity for the Th^T allele,[5] which in single dose produces the thalassemia trait; a double dose of the mutant gene would also be expected to produce a more extreme effect in respect to the A_2 level. However, it must be considered that a third type of hemoglobin, namely hemoglobin F, is present in the thalassemia homozygotes in amounts varying between 20 per cent and 90 per cent. Because hemoglobin F is probably produced through a metabolic pattern in some way independent of the hemoglobin locus, it may be more pertinent to calculate A_2 as per cent of the total hemoglobin other than fetal. The values of A_2 in Th^T/Th^T then approach or surpass those in Th^T/Th^+.

A simple genetic explanation of the observed facts concerning the A_2 hemoglobin is not easily found.

[5] For the thalassemia genes, which are usually regarded as not allelic with Hb^A, Hb^S, Hb^C, the mutant allele is indicated as Th^T (T from thalassemia) and the normal allele as Th^+.

It must be realized that A_2 hemoglobin being present, although at a lower level in normal and Hb^S/Hb^S or Hb^A/Hb^S genotypes, cannot be regarded as a specific product arising from processes controlled by either the hemoglobin alleles or the Th^+ gene. It could be a by-product of the same metabolic steps which involve the hemoglobin alleles, or it could arise from mechanisms controlled by an entirely different locus. In any case, it is of interest to note that the increase of A_2 hemoglobin from the normal level 2.5 per cent to 5 per cent in the Th^T/Th^+ is of the same relative magnitude as the two-fold increase of the S or C hemoglobins in the Hb^A/Hb^S or Hb^A/Hb^C heterozygotes when also a Th^T allele is present in the genotype. The increase of the abnormal S or C hemoglobins from approximately 40 per cent to approximately 80 per cent is explained by regarding the mutant Th^T gene as a modifier of dominance upon the alleles at the hemoglobin locus. In the normal homozygote (Th^+/Th^+) the two alleles, Hb^A and Hb^S, express themselves almost equally in the phenotype. However, when one Th^T is present, hemoglobin S may become completely dominant over hemoglobin A. On the phenogenetic level this means that the metabolic disturbance induced by a Th^T gene acts differentially on the relative rates of synthesis of the normal A and of the abnormal S or C hemoglobins. Similarly, it may be inferred that a Th^T gene, specifically impairing the syntheses of A hemoglobin, raises the level of the normal A_2 hemoglobin in the same way as it modifies the expression of the pathological S and C traits; in other words, the metabolic disturbance induced by a Th^T gene acts differentially on the relative rates of synthesis of the A and A_2 components.

According to the data collected by Bianco, Montalenti, Silvestroni, and Siniscalco (18), the hematological features most useful for discriminating between normal individuals and thalassemia carriers are in the order: the mean corpuscular volume, the osmotic fragility and the red cell count, with a probability of correctness under optimal laboratory conditions of 98.85 per cent, 98.84 per cent, and 91.90 per cent, respectively (19). Analyses of the data obtained in the present study indicate that the efficiency of the A_2 determination (94 per cent) is of the same order. The discriminating usefulness of the standard procedures is somewhat reduced in practice when thalassemia must be recognized not so much against normals but rather against other hematological conditions; for example, deficiency anemias. It seems likely that determination of A_2 hemoglobin will aid in making such distinctions, although further studies are necessary for determining the exact correlation between the A_2 level and the other hematological findings which are typical for the heterozygous thalassemia state.

SUMMARY

The minor hemoglobin component (A_2) of normal blood was readily separated from the main hemoglobin fraction by various procedures of zone electrophoresis. In the isolated form it showed all the usual characteristics of a true hemoglobin. The shifts in the absorption spectrum obtained with various derivatives were similar to those obtained with hemoglobin A. A close resemblance in sedimentation coefficient, solubility, and immunological properties was found for these hemoglobins.

A_2 hemoglobin was found in the blood of all of approximately 300 normal adult individuals but was absent or greatly reduced in newborn infants. A similar component was found in the blood of certain other primates, but not in other animals.

Quantitation of the A_2 component revealed elevated levels in individuals with thalassemia. A mean level of 5.11 ± 1.36 per cent compared to the normal of 2.54 ± 0.35 per cent was obtained for a series of 34 carriers of the thalassemia trait. The highest values were obtained in two patients classified as intermediate forms of thalassemia. Children with Cooley's anemia showed considerable variation with most values falling in the normal range.

The possible value of analyses of the A_2 component in various thalassemia syndromes and their genetic implications are discussed.

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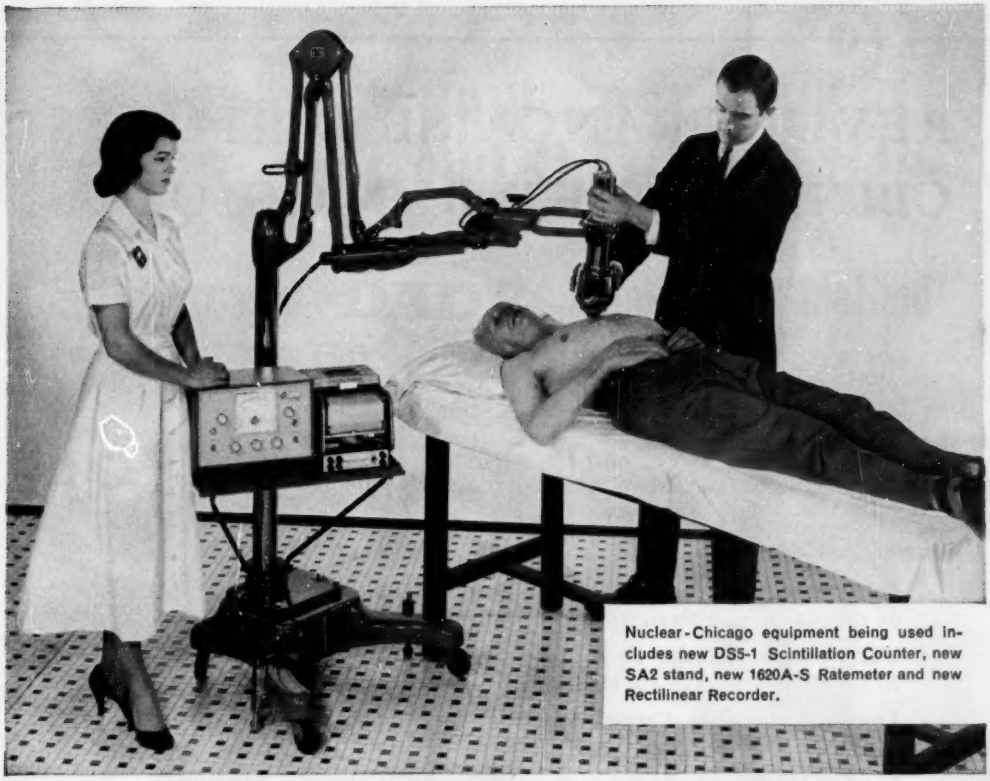

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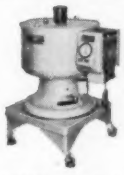

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